Neuronal Mechanisms Involved in Cancer Anorexia-Cachexia Syndrome and Evaluation of Possible Therapeutic Approaches

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“Sono così avanti che quando gli altri mi raggiungono ciaooone”

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1 Summary

The cancer anorexia-cachexia syndrome (CACS) is characterized by reduced eating and body weight loss, which is the consequence of reduced fat and muscle mass. CACS is present in up to 85% of cancer patients and represents the most significant negative predictor of treatment outcome. Importantly, it also deteriorates quality of life and leads to severe psychological suffering of patients and their families. The currently available therapeutic approaches are not sufficiently effective and poorly understood as far as the underlying mode of action is concerned. Therefore, the identification of the underlying pathophysiology and the development of new pharmacological approaches against CACS have a high clinical relevance.

The *area postrema* (AP) and the *nucleus tractus solitarii* (NTS) are two important brainstem centers implicated in the mediation of anorexia, emesis and nausea during sickness conditions. However, the knowledge about the role of the AP and NTS in CACS is very limited**.** Recently evidence suggests that the macrophage inhibitory cytokine-1 (MIC-1) acts as circulating tumor-derived factor promoting anorexia and body weight loss. Using neurosurgical approaches we examined the role of the AP and vagal afferents projecting to the NTS in the mediation of cancer anorexia and body weight loss in a rat hepatoma tumor model. Specifically, we investigated whether a lesion of the AP or subdiaphragmatic vagal deafferentation attenuate anorexia, body weight, muscle and fat loss. Moreover, MIC-1 blood levels were analyzed at different time points during cancer progression. AP lesioned rats were protected against anorexia, body weight loss and muscle atrophy induced by tumor growth. In contrast, the subdiaphragmatic vagal deafferentation did not attenuate cancer-induced anorexia or body weight loss. Tumor-bearing (TB) rats had substantially increased MIC-1 levels, which positively correlated with tumor size and cancer progression, and negatively correlated with food intake. These findings demonstrate the importance of the AP in the central mediation of CACS in our tumor model and support a pathological role of MIC-1 as a tumor-derived factor mediating CACS, possibly via an AP-dependent action.

The glucagon-like peptide-1 (GLP-1) is expressed in neurons of the NTS. GLP-1ergic neurons project from the NTS to the AP where the GLP-1 receptor (GLP-1R) is highly expressed. GLP-1 is implicated in malaise, nausea and aversion induced by different stimuli. Therefore, we hypothesized that GLP-1 signaling in the AP/NTS region also mediates anorexia following tumor growth. We first tested whether a pharmacological blockade of brainstem GLP-1 via chronic delivery of the GLP-1R antagonist exendin-9 (Ex-9) into the fourth ventricle attenuated CACS. Second, we investigated whether a genetic knockdown of GLP-1 expression in the NTS ameliorates CACS. Finally, using a two diets choice paradigm, we tested if tumor growth in our model is paralleled by the development of food aversion. Pharmacological or genetic blockade of brainstem GLP-1 signaling attenuated anorexia, body weight loss and fat depletion. Importantly, Ex-9 treatment was also effective in preventing muscle degradation. Furthermore, TB rats developed tumor-induced food aversion because their flavor preference turned into a flavor rejection during tumor growth. Our study identifies brainstem GLP-1 as crucial mediator of CACS in hepatoma TB rats. In line with the function of GLP-1 as a mediator of aversion, emesis and nausea, we also demonstrated the development of food aversion during CACS in this model. Since the AP lacks a functional the blood-brain-barrier, the GLP-1R located in the AP represents a promising target for pharmacological approaches against CACS.

In addition to brainstem mechanisms, forebrain structures in particular the arcuate nucleus (Arc) seem to be implicated in the mediation of CACS. Hence, the underlying signaling cascades influencing Arc activity represent potential therapeutic targets. The gastric hormone ghrelin positively affects energy balance by increasing food intake and reducing energy expenditure. These effects are mainly mediated via the Arc. Therefore, ghrelin mimetics are another possible treatment against CACS. Using the aforementioned rat hepatoma model, the action of the non-peptidergic ghrelin receptor agonist HM01 energy homeostasis and muscle mass was evaluated. TB rats treated with HM01 had higher food intake than TB controls and were protected against body weight loss and muscle degradation. Moreover, TB rats reduced their metabolic rate following HM01 treatment. These results emphasize the possible therapeutic usefulness of ghrelin receptor agonists like HM01 for the treatment of CACS.

In conclusion, we substantiated the fundamental role of the AP in the mediation of CACS in our tumor model and we provided evidence for a role of central GLP-1 signaling in hepatoma TB rats. Furthermore, we demonstrated the therapeutic efficacy of the novel ghrelin analog HM01 in the context of CACS. Both ghrelin and GLP-1-based approaches represent promising options for the treatment of CACS and possibly other forms of disease-related anorexia.

2 Zusammenfassung

Das krebsbedingte Anorexie-Kachexie-Syndrom (engl. CACS) zeichnet sich durch einen Verlust des Körpergewichts aus, welchem eine verminderte Nahrungsaufnahme und ein erhöhter Fett- und Muskelmasseabbau zugrunde liegt. CACS tritt bei bis zu 85% der Krebspatienten auf und ist der wichtigste Faktor, welcher den Therapieerfolg negativ beeinflusst. Zusätzlich reduziert CACS die Lebensqualität und kann schwere psychische Probleme bei Patienten und deren Familien hervorrufen. Leider sind die derzeit verfügbaren therapeutischen Ansätze nicht wirksam genug und deren Mechanismen unbekannt. Folglich ist die Erforschung der zugrundeliegenden Pathophysiologie sowie die Entwicklung neuer pharmakologischer Ansätze von hoher klinischer Bedeutung.

Die *Area postrema* (AP) und der *Nucleus tractus solitarii* (NTS) sind zwei wichtige Zentren im Hirnstamm, die bei der physiologischen Kontrolle der Nahrungsaufnahme und der Vermittlung krankheitsbedingter Anorexie sowie bei Übelkeit und Erbrechen beteiligt sind. Ihre Beteiligung bei der Entstehung von CACS ist derzeit noch unklar. Als ein wichtiger tumor-assoziierter Faktor wurde kürzlich das *Macrophage inhibitory cytokine-1* (MIC-1) identifiziert, welches Anorexie und Körpergewichtsverlust, wahrscheinlich durch einen direkten zentralen Effekt auslösen kann. Mittels neurochirurgischer Ansätze haben wir die Rolle der AP und der vagalen Afferenzen, die zum NTS projizieren, bei der Vermittlung von CACS anhand eines Ratten Hepatoma-Tumormodells untersucht. Dabei haben wir erforscht, ob eine Läsion der AP oder die Deafferenzierung der subdiaphragmatischen vagalen Afferenzen der krebsbedingten Reduktion von Futteraufnahme, Körpergewicht sowie Muskel- und Fettmasse entgegenwirkt. Ausserdem haben wir die Plasmakonzentration von MIC-1 zu verschiedenen Zeitpunkten während des Tumorwachstums untersucht. Im Gegensatz zu der subdiaphragmatischen vagalen Deafferenzierung hob eine Läsion der AP die tumorbedingte Anorexie und den Körpergewichtsverlust der Ratten auf. Die MIC-1 Plasmakonzentration war bei tumortragenden Ratten signifikant erhöht und korrelierte positiv mit der Tumorgrösse und negativ mit der Nahrungsaufnahme. Diese Ergebnisse zeigen die Wichtigkeit der AP bei der Entwicklung der krebsbedingten Anorexie und Körpergewichtsreduktion in unserem Tumormodell und stützen die Annahme, dass

MIC-1 eine wichtige Rolle bei der Entwicklung von CACS spielt und vermutlich über die AP wirkt.

Das Peptid „Glucagon-like peptide-1“ (GLP-1) wird unter anderem in Neuronen des NTS exprimiert. Diese Neuronen projizieren zur AP, die eine hohe Expression des GLP-1 Rezeptor (GLP-1R) aufweist. GLP-1 spielt eine wichtige Rolle bei der Entstehung von Unwohlsein, Übelkeit und Futteraversion, die durch verschiedene Reize ausgelöst werden können. Wir vermuteten, dass auch die tumorbedingte Anorexie über GLP-1 in der AP/NTS-Region vermittelt werden könnte. Zunächst haben wir untersucht, wie sich eine pharmakologische Blockade vom GLP-1R im Hirnstamm durch Infusion des GLP-1R-Antagonisten Exendin-9 (Ex-9) in den vierten Ventrikel auf das CACS auswirkt. Zusätzlich haben wir getestet, ob eine Reduktion der GLP-1 Genexpression im NTS von Ratten durch einen genetischen GLP-1 „knockdown“ vor Anorexie und Körpergewichtsverlust bei Tumoren schützt. Schlussendlich haben wir getestet, ob das Tumorwachstum in unserem Modell mit der Entstehung einer Nahrungsaversion assoziiert ist. Die pharmakologische sowie auch die genetische Inhibition von GLP-1 im Hirnstamm führte zu verminderter Anorexie und reduziertem Körpergewichts- und Fettverlust. Die Ex-9 Behandlung verminderte zusätzlich den Muskelabbau. Ausserdem entwickelten tumortragende Ratten eine tumorinduzierte Nahrungsaversion gegen das Futter, das sie vor Beginn des Tumorwachstums besonders gern zu sich nahmen. Unsere Studien zeigen somit, dass GLP-1 im Hirnstamm eine entscheidende Rolle bei der Entwicklung von CACS in tumortragenden Ratten spielt. Zusätzlich haben wir in diesem Modell ebenfalls die Entwicklung einer Nahrungsaversion im Zusammenhang mit CACS gezeigt. Da der AP eine funktionelle Blut-Hirn-Schranke fehlt, sind die GLP-1 Rezeptoren in der AP ein möglicher Angriffspunkt für neuartige Therapien gegen CACS.

Zusätzlich zu den Hirnstammmechanismen sind offensichtlich Strukturen im Vorderhirn, insbesondere der *Nucleus Arcuatus* (Arc), an der Vermittlung des CACS beteiligt. Daher stellt auch der Arc ein potenzielles therapeutisches Ziel dar. Das Hormon Ghrelin wirkt sich positiv auf die Energiebilanz aus, indem es die Nahrungsaufnahme erhöht und den Energieverbrauch senkt. Diese Effekte werden hauptsächlich über den Arc vermittelt. Deswegen stellen Ghrelin-Mimetika grundsätzlich eine weitere Behandlungsmöglichkeit gegen CACS dar. Mittels unseres

Tumormodells in Ratten untersuchten wir die Wirkung des Ghrelin-Rezeptor-Agonisten HM01 auf die Energiehomöostase und die Muskelmasse bei Ratten. Tumortragende Ratten, die mit HM01 behandelt wurden, hatten eine höhere Futteraufnahme als die tumortragenden Kontrollen sowie einen geringeren Körpergewichtsverlust und Muskelabbau. Außerdem reduzierten die mit HM01 behandelten Ratten ihre Stoffwechselrate. Diese Ergebnisse legen eine mögliche therapeutische Nutzung von Ghrelin-Rezeptor-Agonisten wie HM01 für die Behandlung von CACS nahe.

Zusammenfassend haben wir die Bedeutung der AP und des NTS bei der Vermittlung des CACS in unserem Tumormodell untersucht und haben dabei GLP-1 als massgeblich für die Signalvermittlung identifiziert. Weiterhin haben wir die therapeutische Wirksamkeit des Ghrelin-Analogs HM01 bei CACS nachgewiesen. Sowohl Ghrelin- als auch GLP-1-basierte therapeutische Ansätze stellen vielversprechende Möglichkeiten für die Behandlung von CACS und eventuell auch für andere Formen von krankheitsbedingter Anorexie dar.

3 Introduction

3.1 The cancer anorexia-cachexia syndrome

3.1.1 Definition

The cancer anorexia-cachexia syndrome (CACS) is a complex multifactorial syndrome that is clinically characterized by reduced eating (anorexia), hypermetabolism and body weight loss, which is the consequence of reduced fat and especially muscle mass. Other symptoms of CACS may include anemia, weakness, nausea, food aversion and depression [1]. The origin of the terms anorexia and cachexia is Greek. Anorexia is the combination of the term “an” (no) and “orexis” (appetite) while the term cachexia is the union of words “kakós” (bad) and “hexis” (condition or appearance). Although anorexia-cachexia has been recognized as a severe medical illness for centuries, there has been no widely accepted definition. Recently a new definition of CACS based on international consensus appeared. CACS is defined as “a multifactorial syndrome characterized by an ongoing loss of skeletal muscle mass (with or without loss of fat mass) that cannot be fully reversed by conventional nutritional support and leads to progressive functional impairment. The pathophysiology is characterized by a negative protein and energy balance driven by a variable combination of reduced food intake and abnormal metabolism” [2].

3.1.2 Epidemiology

CACS affects more than a half million of cancer patients in the US and roughly 1 million in Europe [3]. Nearly 85% of patients with gastric and pancreatic cancers exhibit CACS, whereas CACS was reported in approximately 50% of lung, prostate and colon cancer [4, 5]. On the other hand, less than one third of the patients suffering from acute leukemia and breast cancer developed CACS [6]. Its severity is both age and cancer specific. The greatest body weight loss can be observed in patients with solid tumors, in

particular in lung, gastric and pancreas cancer patients. Furthermore, CACS is more common in children and elderly patients and becomes more pronounced as the disease progresses [7]. At the time of diagnosis (i.e. before any treatment), 80% of patients with upper gastrointestinal cancers and 60% of patients with lung cancer have already experienced substantial weight loss [5].

3.1.3 Clinical relevance

CACS is associated with reduced survival. Patients who show prolonged weight loss over time have a significantly worse prognosis [1, 5]. This is presumably the consequence of increased susceptibility to infections, decreased tolerance to anticancer therapies and lack of essential micronutrients (e.g. iron) [8]. In particular, cancer cells of those patients are more resistant to cancer-therapies, requiring higher doses of chemotherapeutic agents. Unfortunately, CACS is associated with an increased prevalence of chemotherapeutic side effects, leading to necessary dose reductions, treatment delays or even treatment abortion. Thus, patients suffering from CACS do not receive the full potential benefit of the actual cancer therapy [8-10]. The mortality rate of patients with CACS reaches up to 80% per year [3]. CACS accounts for 20% of cancer-related deaths i.e., severe cachexia was the only symptom present at the time of death [11]. It is estimated that each year CACS contributes to more than 7 million of deaths worldwide [12]. Notably, CACS also negatively impacts quality of life. Independency and physical activity are severely compromised due to muscle wasting and the involuntary body weight loss affects patients' self-esteem and identity [13, 14]. Furthermore, the loss of appetite and nausea causes psychological distress and is associated with increased apathy, which further decreases life quality [15].

3.1.4 Current therapeutic options

The effectiveness of both nutritional care and pharmacological treatments against CACS is unfortunately very limited and insufficient progress has been made in the development of specific pharmacological approaches [8]. Hypercaloric feeding

increases body weight but does not affect lean body mass or survival, indicating that nutritional counseling alone is an inadequate strategy [16]. No approved drug for the treatment of CACS is currently available and, despite its severity, only few large-scale human trials have been conducted. The most commonly used agent, the appetite stimulating drug megestrol acetate, only moderately increases eating and body weight. However, its body weight gaining effect is mainly due to water retention and not to increased lean mass [17]. Corticosteroids are another class of pharmacological agents used for the treatment of CACS. Although they do not promote body weight gain, they improve appetite, decrease inflammation and increase performance status. Unfortunately, the beneficial effects of corticosteroids are not long lasting, as they seem to disappear after 4 weeks of treatment. Furthermore, long-term use of these agents induces severe side effects. Thus, corticosteroids are not suitable for long-term use and recommended to be used only during the terminal phase of cachexia [8, 18]. Other drugs such as cannabinoids and anti-inflammatory agents, despite high expectations, have failed to show tangible benefits compared to megestrol acetate or placebo, respectively [19, 20]. A vast spectrum of other treatments, including androgens, serotonin and histamine receptor antagonists, has been studied in the past without much success or they have not been developed into effective therapies yet [21]. In general, medical use of the aforementioned treatments is very limited due to side effects (adrenal insufficiency, thromboembolism, insulin resistance, neurologic effects, sexual dysfunction etc.). Moreover, these drugs do not appear to significantly improve survival and general quality of life in most of the clinical trials. Therefore, the identification of the pathophysiological mechanisms mediating CACS and the development of new pharmacological approaches that simultaneously tackle different aspects of this syndrome (i.e. anorexia, inflammation, hypermetabolism), has a high clinical relevance.

3.2 The regulation of energy homeostasis under physiological conditions

Living organisms rely on energy intake to fulfill their biological functions. The maintenance of energy homeostasis, i.e. the balance between energy intake (calories consumed) and energy expenditure, is essential to maintain a stable body weight over

long period of time [22]. An imbalance between these two variables may result in increased or decreased energy deposits (primarily fat). If this disequilibrium lasts over a long period of time, it may lead to negative consequences for the organism (e.g. cachexia, obesity). Feeding in humans and in many animals is organized into discrete meals. Meal initiation, termination, size and frequency are regulated by signals that are triggered in response to nutrient ingestion (e.g. satiation signals) as well as signals regarding the status of the energy stores (i.e. adiposity signals), which upon peripheral release, are sensed and integrated by the central nervous system (CNS) [22, 23].

Most satiation signals are peptides released from the gastrointestinal tract (GI) in response to nutrient ingestion. These signals include the gut hormone cholecystokinin (CCK) and the pancreatic hormone amylin. After being released, they can either reach their central site of action directly via the bloodstream or act in a paracrine manner on nerve afferents that innervate the GI. For instance, the anorectic effect of CCK is mediated via specific receptors on afferent vagal fibers that innervate the gut. On the other hand, amylin acts centrally by directly stimulating its receptors expressed in the *area postrema* (AP) of the brainstem [24-26]. The satiating signals acting on the AP and on vagus nerve terminals are both transmitted to the *nucleus tractus solitarii* (NTS), another important brainstem nucleus involved the control of eating [27].

Adiposity signals are hormones secreted in proportion to body fat thus providing long-term information about the energy source of the body. The best-characterized adiposity signals are leptin, which is secreted by adipocytes, and the pancreatic hormone insulin. [24-26]. Both leptin and insulin act via the arcuate nucleus (Arc) to control feeding behavior. Leptin and insulin exert their direct effects on feeding by stimulating pro-opiomelanocortin (POMC) neurons, which express the anorexigenic transmitters α-melanocyte stimulating hormone (αMSH) and cocaine- and amphetamine-regulated transcript (CART), and by inhibiting neurons releasing the orexigenic neuropeptides agouti-related peptide (AgRP) and neuropeptide Y (NPY) [28-31].

In addition to adiposity signals, the hunger hormone ghrelin also exerts its eating-promoting effects mainly via the Arc. Ghrelin is the best-characterized peripheral orexigenic peptide [32]. It is secreted by the stomach shortly before meals and functionally counteracts leptin and insulin by activating NPY neurons and inhibiting POMC neurons [28, 30, 31, 33].

3.3 The pathophysiology of CACS

Tumor growth is associated with profound metabolic and neurochemical alterations, which can lead to the development of CACS. The severe loss of body weight observed in cancer patients is due to a combination of decreased energy intake and increased energy expenditure. In addition, increased muscle protein catabolism leads to severe muscle degradation [6]. Thus, CACS fundamentally differs from simple starvation characterized by increased appetite, reduced energy expenditure and loss of fat but not lean mass [34]. Many of the processes contributing to CACS are mediated by humoral factors acting centrally on brain areas involved in the control of energy balance and acting peripherally on muscles [1, 35].

Among other factors, inflammatory cytokines released by the tumor-itself or by the host immune system play a major role in development of CACS [36]. The role of cytokines as mediators of anorexia was established in models of acute inflammation, e.g. after treatment with the bacterial endotoxin lipopolysaccharide (LPS) [37]. Furthermore, acute and chronic administration of different cytokines, such as TNF-α, IL-6, IL-1β and INF-γ, either alone or in combination, are capable of reducing food intake and inducing body weight loss [35-38]. In addition to these classical inflammatory cytokines, the recently discovered macrophage inhibitory cytokine-1 (MIC-1) also induces anorexia and body weight loss [39]. The anorectic effects of cytokines were also observed after central cytokine injections, suggesting a direct involvement of the brain [40-42]. Indeed, several cytokine receptors (including IL-1β, TNF-α and IFN-γ) are expressed in the brainstem and the hypothalamus [43, 44]. Moreover, cytokine treatment leads to neuronal activation in brain regions controlling energy homeostasis, as determined by the detection of the marker c-Fos [45-47]. Circulating cytokines may reach brain areas involved in the control of eating via active transport or through regions of the brain lacking a functional blood-brain barrier (BBB) [37, 38, 43, 48, 49]. In addition to this humoral pathway, vagal afferent signaling can also contribute to the central transmission of cytokines effects [50, 51].

The activation of cytokine receptors causes alterations of a wide range of intracellular signaling cascades inducing changes in gene transcription and in neuronal activity [51]. For instance, the signal transduction of different cytokines involves the phosphorylation and activation of transcription factors of the STAT family (signal transducers and activators of transcription) [52]. The leptin intracellular signaling cascade also involves STAT signaling, suggesting that cytokines induce effects on energy homeostasis that mimic leptin in some regards, and that the increased hypothalamic actions may contribute to anorexia and body weight loss [35, 53]. Moreover, cytokines interact with endothelial cells of the BBB to activate the transcription factor NF-κB [54, 55] that induces the expression of prostanoids and nitric oxide (NO) [56, 57]. Both NO and prostanoid signaling cascades play an important role in transducing cytokine actions into a modulation of neuronal activity [50].
Similar to LPS injections, tumor growth induces peripheral and central expression of different inflammatory cytokines in many animal models for CACS [58-61]. Moreover, neutralization of cytokine actions with specific antibodies partly attenuates anorexia and body weight loss [60, 62-64]. High serum levels of TNF-α, IL-6, IFN-γ, IL-1β and MIC-1 also occur in cancer patients, and the levels of these cytokines correlate with cancer progression [65-70].

Cytokines also contribute to the etiology of CACS by direct peripheral actions on muscles [71, 72]. Increased inflammatory signaling negatively affects muscles by suppressing protein synthesis and increasing protein degradation [6, 73]. TNF-α, IL-1β, IFN-γ and IL-6 have all been implicated in the mediation of muscle degradation during cancer [73]. The ubiquitin-proteosome pathway is one of the main pathways regulating muscle degradation [74]. Cytokines released during tumor growth activate the ubiquitin-proteosome pathway in many animal models as well as in humans [73, 75, 76]. In particular, the E3 ligases muscle atrophy F-box protein (MAFbx/atrogin-1) and muscle RING finger protein-1 (MURF-1) are highly upregulated during cancer conditions [77-80], and pharmacological blockade of ubiquitin-proteosome pathway effectively reduces muscle atrophy in animal models for CACS [81, 82].

The overall aim of the thesis is to shed more light on the neuronal mechanisms involved in CACS and to evaluate possible therapeutic strategies. Thus, the putative brain structures, as well as the underlying neurotransmitter involved will be discussed in

details in the following chapters.

3.4 Brainstem mechanisms contributing to sickness anorexia and nausea

3.4.1 The area postrema

The AP is a component of the dorsal vagal complex, a major viscerosensory and autonomic center of the brainstem. The AP is located on the dorsal surface of the medulla oblongata [83]. It belongs to a group of brain structures called sensory circumventricular organs (sCVO). sCVO are specialized brain structures situated near the ventricular spaces in the midline of the brain. They are densely vascularized and lack a functional BBB. In mammals, sCVO include the AP, the subfornical organ and the vascular organ of lamina terminalis [84]. Due to the fenestrated capillaries, neurons of the sCVO can be reached by humoral factors that cannot cross the BBB in other brain areas [85-87]. The AP is reciprocally connected to a variety of different nuclei. In particular, neurons located in the AP send reciprocal axonal projections to NTS, lateral lateral parabrachial nucleus (LPBN) and dorsal motor nucleus of the vagus (DMN) [83, 88].

Several studies demonstrated the role of the AP in the autonomic control of different physiological processes including feeding, metabolism, fluid balance and the cardiovascular system [87]. The AP mediates the anorexigenic effect of the hormone amylin, which is co-secreted with insulin by β pancreatic cells during and after food intake [89-91]. The AP was also functionally described as a chemoreceptor trigger zone mediating emetic reflexes (e.g. vomiting, retching and nausea) induced by noxious chemical stimuli [92, 93]. These effects appear to be mediated by specific neuronal subpopulations that are distinct from neurons transducing amylin's effects or regulating blood pressure [94]. Ablation of the AP prevents vomiting in response to many different emetic stimulants (e.g. radiation, chemotherapeutic agents) in different animals including humans [95, 96]. Different studies demonstrated the involvement of the AP in sickness-related anorexia. In fact, neuronal activation has been shown in the AP after

immunological challenge with LPS, IL-1β and MIC-1 [97-99]. AP lesions attenuate TNF-α and MIC-1 induced anorexia and body weight loss [100, 101].

3.4.2 The nucleus of the solitary tract

The AP is anatomically and functionally linked with the adjacent NTS [102, 103]. The caudal part of the NTS receives and processes vagal afferent signals that control food intake, including mechanical (i.e. volumetric distension), hormonal (e.g. CCK) and chemical stimuli (e.g. glucose, amino acids, toxins) [27, 104]. In addition to the AP, the NTS is connected to other brain areas that are relevant for the control of food intake including the Arc, LPBN, DMN, paraventricular nucleus (PVN) and lateral hypothalamic area [104, 105]. Similar to the AP, the NTS has a crucial role in the mediation of food aversions by integrating and processing noxious-related vagal afferent signals such as the chemotherapeutic agent cisplatin, the toxin lithium chloride (LiCl) and the mucosal irritant $CuSO_4$ [27, 106-110]. Hence, together with the AP, the NTS represents an important site of convergence for humoral and neuronal signals that are transmitted to other rostral brain centers relevant for the physiological and pathological control of energy balance [102, 103].

3.4.3 The role of the AP and NTS in the mediation of CACS

Despite importance of the AP and NTS in the mediation of a wide range of pathological and noxious stimuli, there is little experimental evidence for a role of the AP or NTS in cancer anorexia. AP/NTS neurons are activated in tumor-bearing (TB) rats carrying hepatoma tumor and the degree of activation correlated positively with tumor growth and negatively with body weight gain [111]; thus suggesting a role of this area in the central mediation of CACS. However, the only study specifically addressing the role of AP/NTS was conducted using a rat Leydig cell tumor model, which is characterized by high circulating estrogen levels [112]. Given that estrogens act via the AP/NTS region to reduce eating [113], the results of that study possibly reflected the anorectic effects of estrogens rather than specific tumor-dependent signaling mechanisms. Moreover, the

surgical approach utilized to ablate the AP also lesioned major parts of the NTS resulting in a partial destruction of vagal terminal fields [114]. Therefore, knowledge about the specific contribution of the AP in the mediation of CACS is very limited and a better dissociation between vagal and AP-dependent mechanisms is required. In addition, the putative mechanism leading to AP/NTS activation needs deeper investigation. Given that important anatomical and functional link between the AP and NTS it is logical to assume that neuropeptide system within this region could mediate the presumed effects on energy homeostasis during cancer.

3.4.4 Glucagon-like peptide-1

Glucagon-like peptide-1 (GLP-1) is a peptide produced from the precursor pre-pro-glucagon (PPG), which is encoded by the *Gcg* gene. GLP-1 is released by L-cells located in the mucosa of the gastrointestinal tract within a few minutes after meal initiation [115-118]. GLP-1 was initially identified as an incretin hormone for its ability to potentiate glucose-induced insulin secretion [119]. Subsequent studies demonstrated the importance of GLP-1 in the regulation of eating; particularly in meal termination (i.e. inducting satiation). Similarly to CCK, intestinal GLP-1 reduces meal size mainly acting in a paracrine fashion, activating the vagal afferent terminals located adjacent to the site of release [120]. However, both central and peripheral GLP-1 administration reduce food intake and body weight [121-124]. At least when infused into the hepatic portal vein, pharmacological doses of GLP-1 inhibits eating via direct action on brain areas important for the control of energy homeostasis [125]. GLP-1 activates the GLP-1 receptor (GLP-1R), a 7-transmembrane G protein-coupled receptor [126]. In addition to vagal afferent terminals, GLP-1R expression has been identified in many brain areas relevant for the control of food intake and energy homeostasis including the AP [119, 127-130].

GLP-1 is also produced in the central nervous system, specifically in enteroceptive neurons located in the caudal NTS [127]. Central GLP-1 is released from axon terminals of GLP-1 expressing NTS neurons and acts as a peptidergic neurotransmitter. GLP-1-expressing neurons project to most of the known GLP-1R-expressing brain areas [131,

132]. Notably, GLP-1 positive terminals have also been identified in the AP [133] suggesting that brain-intrinsic GLP-1 may act on the AP.

3.4.5 Involvement of central GLP-1 in the mediation of sickness anorexia and nausea

Besides its presumed role in the control of eating, the central GLP-1 system is also involved in the mediation of malaise and in the development of food aversions. GLP-1 or the GLP-1R agonist exendin-4 (Ex-4) induce emesis and nausea in humans [134-136] and conditioned taste aversion (CTA) in rodents [137-139]. Moreover, the central GLP-1 system mediates CTA and malaise induced by LiCl and cisplatin as demonstrated by a blockade of CTA and anorexia by fourth ventricular administration of the GLP-1R antagonist exendin-9 (Ex-9) [140-142]. These findings are consistent with immunohistological studies showing a blockade of LiCl-induced AP activation by third ventricular Ex-9 treatment [137]. Furthermore, LiCl and cisplatin activate GLP-1 expressing NTS neurons [142, 143]. There is also evidence for a role of central GLP-1 signaling in the mediation of the anorectic effect of LPS. LPS induces the expression of c-Fos in the AP/NTS region [143]. Similar to what has been described for LiCl and cisplatin [137, 142], LPS-induced anorexia is attenuated in rats receiving central injection of Ex-9 [144]. Interestingly, this effect is only observed when Ex-9 was injected into the fourth ventricle. In contrast, no effects of Ex-9 treatment on LPS anorexia were noticed after third ventricular injection, suggesting that the brainstem GLP-1 signaling (i.e. within the AP/NTS) plays a predominant role in the mediation of sickness-induced anorexia [144]. However, the contribution of the central GLP-1 signaling in the central mediation of CACS still remains unknown. Furthermore, the upstream mediator responsible for the activation of the AP/NTS region still needs to be identified.

3.4.6 The macrophage inhibitory cytokine-1 as a potential peripheral mediator of CACS via AP/NTS signaling

MIC-1 also known as growth differentiation factor 15 (GDF15) belongs to the TGF-β superfamily. Under physiological conditions, MIC-1 is expressed at low levels in different tissues and is present at low concentration in the blood stream [145]. During cancer, tumor-dependent expression of MIC-1 can increase serum levels by up to 100 fold [146-148]. Several clinical studies support its potential use as a biological marker for prognosis and diagnosis of certain types of cancer [39, 70, 149, 150]. Interestingly, circulating MIC-1 levels often correlate with the severity of CACS, pointing to a clinical relevance of MIC-1 as a possible mediator of CACS. Mice overexpressing MIC-1 have a leaner phenotype and show reduced food consumption [151]. Conversely, MIC-1 knockout mice show increased adiposity [152]. More importantly, MIC-1 produced by xenografted tumors and recombinant protein both reduce food intake and lead to body weight loss in mice, which can be attenuated by specific monoclonal antibodies against MIC-1 [99]. In mice treated with MIC-1, the expression of c-Fos in the AP/NTS is increased [99, 101]. In line with these observations, lesion of the AP/NTS prevents anorexia and body weight loss induced by chronic MIC-1 treatment suggesting that MIC-1 may act via this brain region to inhibit food intake and body weight [101].

3.5 Hypothalamic and peripheral mechanisms that can be target by ghrelin-based anti-CACS treatments

In addition to the brainstem, forebrain structures in particular the Arc seem to be implicated in the mediation of CACS. Therefore, the underlying signaling cascades represent potential therapeutic targets.

3.5.1 The arcuate nucleus

The Arc is an important brain structure for the control of food intake and energy balance [153]. It is located in the ventral hypothalamus around the basal part of the third ventricle, in close proximity to the median eminence [154]. Two subpopulations of Arc neurons exert opposite effects on food intake and energy balance. Neurons co-expressing NPY and AgRP [155] promote feeding, decrease energy expenditure and

body weight gain, and neurons co-expressing POMC and CART inhibit feeding and stimulate energy expenditure [156]. POMC is a precursor of α-MSH, which reduces food consumption acting on melanocortin 4 receptors (MC4R) [157]. AgRP is an endogenous antagonist of MC4R and antagonizes the anorectic activity of POMC neurons [158]. NPY promotes eating [159] acting on Y receptors and inhibiting POMC neurons [160]. Both populations of neurons project to other hypothalamic areas such as the PVN and the lateral hypothalamic area [22, 161, 162].

3.5.2 The role of the Arc in the pathophysiology of CACS

NPY expression in the Arc as well as the amount of NPY released into the PVN are reduced in certain rodent models for CACS [163, 164]. Furthermore, the orexigenic effect of central intra-hypothalamic injection of NPY is blunted in rats bearing methylcholanthrene-induced sarcoma [165]. Interestingly, MC4R knockout mice and rodents receiving central infusion of AgRP or its analog SHU9119 are protected against CACS [166-168]. The effects on Arc NPY and POMC neurons observed following tumor growth are likely mediated by central direct cytokine actions. Indeed, IL-1β administered into the third ventricle antagonizes NPY-induced feeding and decreases NPY mRNA expression at doses that mimicked the cerebrospinal fluid concentrations observed in TB rats [42, 169, 170]. Further, IL-1β, MIC-1 and Leukaemia inhibitory factor stimulate POMC neurons causing increased α-MSH release [53, 99, 171]. Overall, these data indicate that, at least in some tumor models, cytokine signaling in the Arc plays a role in the pathophysiology of CACS, suggesting that substances that counteract or override cytokine actions in the Arc or promote orexigenic Arc signaling may be therapeutically useful as anti-CACS treatment. A potential candidate is the gastric hormone ghrelin.

3.5.3 The role of ghrelin in the control of food intake and energy homeostasis

Ghrelin is a peptide hormone, which is secreted into the circulation by gastric endocrine cells, i.e. X/A-like cells in rodents and P/D1 cells in humans [172-174]. Ghrelin is

encoded by the preproghrelin gene [175]. Circulating ghrelin levels rise pre-prandially and decreases after a meal [176]. Ghrelin levels increase under food restriction and decrease in proportion to the amount of nutrients ingested [32, 177-179]. Ghrelin is present in the circulation in two major forms: active (i.e. acylated at its third amino acid) and inactive (i.e. desacylated). Active ghrelin accounts for 20% of the total ghrelin, while the major fraction of ghrelin circulates in its inactive form [172]. The half-life of active ghrelin is between 8-9 minutes in rats and 13-24 minutes in humans [180-182]. The name "ghrelin" is derived from the Proto-Indo-European "ghre" which is the root of "grow". The name was chosen to indicate its ability to stimulate growth hormone release that together with the ability to promote eating, is the major and best-characterized effect of ghrelin [172].

In various electrophysiological and immunohistological studies, ghrelin has been shown to activate NPY neurons, which is considered the neuronal correlate for ghrelin's actions on food intake and bodyweight. The ghrelin receptor, also known as growth hormone secretagogue receptor (GHSR) is highly expressed in the Arc [183]. 94% of the NPY neurons in the Arc co-express GHSR and belong to the primary target cells for ghrelin in the brain [184, 185]. Peripheral administration of ghrelin induces c-Fos expression in NPY neurons in the Arc [186]. Moreover, ghrelin also increases the mRNA expression of NPY and AgRP in these neurons [187]. As shown by electrophysiological studies, ghrelin directly activates leptin-inhibited Arc neurons [188], while it indirectly (presynaptically) inhibits POMC neurons [189, 190]. Pharmacological and genetic inhibition of NPY neurons abolished the effects of ghrelin [191]. Altogether these data substantiate the key role of the Arc in the mediation of ghrelin effects on eating.

As amply demonstrated, ghrelin decreases energy expenditure, and increases fat accumulation [177]. Chronic central ghrelin treatment increases fat deposition in pair-fed animals suggesting that ghrelin increased adiposity independently of its effect on food intake [192]. This lipogenic effect is in part mediated via the sympathetic nervous system that eventually alters the expression of various enzymes that promoted fat storage [192, 193]. In addition, ghrelin also suppresses the sympathetic outflow to brown adipose tissue (BAT), leading to a reduction in uncoupling protein-1 expression (UCP-1) and eventually a decrease in the amount of energy invested in heat production [194, 195]. Ghrelin plays also an important role in the regulation of glucose homeostasis. Acute administration of ghrelin induces hyperglycemia by a mechanism

that involved reduced insulin secretion by the pancreas and possibly increased hepatic gluconeogenesis [32, 196, 197]. Altogether these effects lead to a reduction of energy expenditure and a higher carbohydrate vs. fat utilization, promoting a positive energy balance independently of ghrelin's orexigenic effect. Due to its positive biological actions on short and long-term energy homeostasis, ghrelin-based approaches represent a potential strategy for the treatment of CACS.

3.5.4 The therapeutic usefulness of ghrelin and its analogs against CACS

In addition to its effect on energy homeostasis, ghrelin has several other actions that might be beneficial for the treatment of CACS. Ghrelin exerts anti-inflammatory effects reflected by its ability to reduce LPS-induced of production the pro-inflammatory cytokines IL-6, TNF-α and IL-1β [198]. Interestingly, ghrelin also inhibits NF-kB signaling in endothelial cells under in vitro conditions [199]. Besides its anti-inflammatory action, ghrelin increases the release of growth hormone (GH) and insulin-like growth factor-1 (IGF-1), which are potent stimulators of a various anabolic pathways leading to muscle growth [172, 200, 201]. GH and IGF-1 signaling in chronic cachectic patients are often impaired mainly due to decreased circulating levels of both GH and IGF-1 [202-204]. Hence, ghrelin's ability to increase GH and IGF-1 levels and to attenuate inflammation could also contribute to the positive effects of ghrelin-based anti-CACS treatments.

Ghrelin and ghrelin receptor agonists have been tested in a number of rodent models for CACS. Most of these studies show positive effects, such as increased food consumption, attenuation of body weight loss and decreased muscle atrophy. A literature overview about these published studies is presented in table 1. However, despite the positive outcome of these preclinical studies, data regarding ghrelin-based treatment in cancer are scarce (see table 1). In fact, there is currently no ghrelin mimetic approved for the treatment of CACS. The orally active ghrelin agonist anamorelin was recently tested in phase III clinical trials for the indication of lung cell cancer CACS. In patients with advanced non-small-cell lung, anamorelin lead to body mass increase and significantly improved appetite and quality of life, without severe

side effects. However, anamorelin did not positively affect handgrip strength, which was one of the primary endpoints in the clinical trial [205].

The clinical use of ghrelin is limited because it has a short half-life and it can only be administered parenterally. Ghrelin mimetics represent a valuable alternative to overcome these limitations. The small non-peptidergic GHSR agonist HM01 belongs to a new generation of orally active ghrelin mimetics. It has high receptor binding affinity, a much higher brain permeability compared to anamorelin and a higher plasma half-life compared to ghrelin [206]. However, its beneficial effects on food intake, body weight, muscle and metabolic rate had not yet been characterized under cancer conditions, which is an aim of the current work.

	Agent	Application	Duration	Species	Underlying model/disease	Main Effects	References
Cancer/chemotherapy	Ghrelin	Daily (b.i.d.) inj., ip.	6 days	Mice	Human melanoma cells	FI + , BW +	Hanada et al.
	Ghrelin	Daily (b.i.d.) inj., ip.	10 days	Mice	Sarcoma	FI + , total and fat mass +	Wang et al.
	Ghrelin, BIM-28131	Chronic (minipumps), sc.	5 days	Rats	Sarcoma	FI + , BW +	DeBoer et al.
	Ghrelin	Daily (d.i.b) inj. ip.	14 days	Mice	Lewis lung carcinoma	muslce atrophy - , inflammation -	Chen al.
	Ghrelin	Single dose, iv.	1 day	Humans	Melanoma, breast, colon cancers	Calories consumption +	Neary et al.
	Ghrelin	2 applications, iv.	2 weeks	Humans	Different cancer types	No differences, no side effects	Strasser et al.
	Ghrelin	Daily, sc.	8 weeks	Humans	Multiple GI malignancies	Appetite + , BW loss -	Lundholm et al.
	Anamorelin	Daily, oral	12 weeks	Humans	Different cancer types	Total and lean body mass +	Garcia et al.
	Anamorelin	Daily, oral	12 weeks	Humans	Lung cancer	Appetite + , BW + , Lean body mass +	Takayama et al.
	Anamorelin	Daily, oral	12 weeks	Humans	Lung cancer	Appetite + , BW + , Lean body mass +	Temel et al.
	Ghrelin	Single dose (rats), 2x (mice), ip.	1 day	Rats, mice	Chemotherapy (cisplatin)	FI + , gastric empting +	Liu et al.
	Ghrelin	Single dose, icv.	1 day	Ferrets	Chemotherapy (cisplatin)	Vomiting and retches -	Rudd et al.
	Ghrelin	Daily inj., ip.	2 weeks	Rats	Chemotherapy (cisplatin)	Anorexia - , BW loss -	Garcia et al.
Other chronic diseases	Ghrelin	Daily inj., iv.	3 weeks	Humans	COPD	FI + , BW + , lean body mass +	Nagaya et al.
	SUN11031	Daily inj., sc., (b.i.d)	3 months	Humans	COPD	BW + , lean body mass +	Gertner et al.
	Ghrelin	Daily inj., sc.	3 weeks	Rats	Coronary artery ligation	FI + , BW loss -	Nagaya et al.
	Ghrelin	Daily inj., iv.	3 weeks	Humans	Chronic heart failure	Lean mass + , heart function +	Nagaya et al.
	Ghrelin	Single dose, iv.	1 day	Humans	Chronic heart failure	Heart function +	Nagaya et al.
	Ghrelin, BIM-28131, BIM-28125	Chronic (minipumps), sc.	2 weeks	Rats	Chronic kidney disease	BW + , lean mass +	DeBoer et al.
	Ghrelin	Single dose, sc.	1 day	Humans	Dialysis dependent renal failure	FI +	Wynne et al.
	Ghrelin	Daily inj., sc.	1 week	Humans	Malnourished dialysis patients	FI +	Ashby et al.
	HM01	Single dose, ip.	1 day	Rats	Costipation	FI + , gastric empting + , + fecal output	Karasawa et al.
Aging	Capromorelin	Daily, oral	1 year	Old adults	Age-related lean mass loss	Lean body mass +	White et al.
	MK-677	Daily, oral	6 months	Old adults	Hip fracture	Extremity functional performance +	Bach et al.

Table 1: Use of ghrelin and its analogs for the treatment of different diseases. *b.i.d: twice a day; BW: Body weight; COPD: chronic obstructive pulmonary disease; FI: food intake; GI: gastrointestinal; +: increase; -: decrease*

4 Aims of the thesis

4.1 Aim 1: the role of the AP/NTS in the mediation of CACS

The AP/NTS may be involved in the mediation of CACS [111]. Using a rat hepatoma tumor model, we examined the role of the AP and of vagal afferents in the mediation of cancer CACS. It was an aim to investigate whether a lesion of the AP (APX) or subdiaphragmatic vagal deafferentation (SDA) attenuate cancer anorexia, and body weight and muscle mass loss. The tumor-derived cytokine MIC-1 emerged as a possible mediator of cancer anorexia [99]. Given the recent evidence for a hindbrain-dependent suppression of food intake by MIC-1 in CACS [101], MIC-1 blood levels were analyzed at different time points during cancer progression. In addition, the impact of tumor growth on metabolic rate, respiratory exchange ratio (RER) and locomotor activity was assessed. Finally, using pair-fed rats we dissociated the effects of tumor anorexia on body weight, body composition and muscle loss from eating-independent effects. For these experiments, the Morris-7777 hepatoma rat model was used for the following reasons: 1) the AP/NTS region is activated following tumor induction; 2) anorexia is a major contributor of CACS in this cancer model.

4.2 Aim 2: the involvement of the central GLP-1 in the mediation of CACS

GLP-1 signaling within the AP/NTS region is an important mediator of aversion and malaise induced by LiCl, LPS and cisplatin [137, 142, 144]. Moreover, GLP-1 signaling in those areas is required for the mediation of the LPS and cisplatin-induced depression of food intake [142, 144]. Therefore, we assumed that GLP-1 signaling in the AP/NTS region may also mediate anorexia following tumor growth.

By taking advantage of both pharmacological and genetic approaches, we investigated whether central GLP-1 signaling is required for the mediation of CACS. First, chronic fourth ventricular infusion of the GLP-1 antagonist Ex-9 was performed and its ability to attenuate CACS was assessed. Second, in order to confirm the involvement of GLP-1 expressing NTS neurons as crucial mediator of CACS, lentiviral vectors expressing shRNA directed against the PPG were bilaterally injected in the NTS. The effect of the genetic GLP-1 blockade on food intake, body weight was measured prior to and after tumor induction. Third, due to the role of the central GLP-1 signaling in the mediation of food aversions, we tested for the presence of tumor-induced diet aversion in our model.

4.3 Aim 3: the effects of the ghrelin analog HM01 as a treatment against CACS

Recently, ghrelin mimetics emerged as possible treatment against CACS. The usefulness of native ghrelin as a therapeutic agent is limited due to its peptidergic nature and its short half-life time. The development of small synthetic GHSR agonists that have longer half-life and high oral bioavailability is a crucial step for future clinical uses. The ghrelin mimetic HM01 has high receptor binding affinity, high brain permeability and a higher plasma half-life compared to ghrelin.

The overall aim of this project was to evaluate the possible usefulness of HM01 against tumor-dependent anorexia and body weight loss. First, we examined the effects of chronic HM01 treatment on muscle mass and body composition in healthy non-tumor-bearing rats. Second, the same rat hepatoma model was used to test whether HM01 ameliorates tumor anorexia, body weight, fat and muscle loss, and whether it affects nutrient utilization and metabolic rate.

5 Original Research Article: “Anorexia-cachexia syndrome in hepatoma tumor-bearing rats requires the area postrema but not vagal afferents and is paralleled by increased MIC-1/GDF15”

The following section contains an original research article that was accepted for publication by the Journal of Cachexia, Sarcopenia and Muscle in October 2016.

My contribution to this publication includes: conception and design of research, data acquisition, data analysis, data interpretation and writing the manuscript.

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Anorexia-cachexia syndrome in hepatoma tumour-bearing rats requires the area postrema but not vagal afferents and is paralleled by increased MIC-1/GDF15

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Abstract

Background The cancer-anorexia-cachexia syndrome (CACS) negatively affects survival and therapy success in cancer patients. Inflammatory mediators and tumour-derived factors are thought to play an important role in the aetiology of CACS. However, the central and peripheral mechanisms contributing to CACS are insufficiently understood. The area postrema (AP) and the nucleus tractus solitarii are two important brainstem centres for the control of eating during acute sickness conditions. Recently, the tumour-derived macrophage inhibitory cytokine-1 (MIC-1) emerged as a possible mediator of cancer anorexia because lesions of these brainstem areas attenuated the anorectic effect of exogenous MIC-1 in mice.

Methods Using a rat hepatoma tumour model, we examined the roles of the AP and of vagal afferents in the mediation of CACS. Specifically, we investigated whether a lesion of the AP (APX) or subdiaphragmatic vagal deafferentation (SDA) attenuate anorexia, body weight, muscle, and fat loss. Moreover, we analysed MIC-1 levels in this tumour model and their correlation with tumour size and the severity of the anorectic response.

Results In tumour-bearing sham-operated animals mean daily food intake significantly decreased. The anorectic response was paralleled by a significant loss of body weight and muscle mass. APX rats were protected against anorexia, body weight loss, and muscle atrophy after tumour induction. In contrast, subdiaphragmatic vagal deafferentation did not attenuate cancer-induced anorexia or body weight loss. Tumour-bearing rats had substantially increased MIC-1 levels, which positively correlated with tumour size and cancer progression and negatively correlated with food intake.

Conclusions These findings demonstrate the importance of the AP in the mediation of cancer-dependent anorexia and body weight loss and support a pathological role of MIC-1 as a tumour-derived factor mediating CACS, possibly via an AP-dependent action.

Keywords Cancer; Food intake; Energy balance; Brainstem; Muscle; AP-lesion; Subdiaphragmatic vagal deafferentation

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Introduction

The anorexia-cachexia syndrome (ACS) is characterized by reduced eating and body weight loss, which is the consequence of reduced fat and muscle mass.[1] More than 80% of patients with advanced cancer suffer from loss of appetite and body weight.[2] Cancer ACS (CACS) negatively affects the clinical status, increases mortality, deteriorates treatment success, and reduces quality of life.[3,4] Inflammatory signalling molecules (e.g. cytokines) that are released during malignancy by the

host immune system or by the tumour itself are important mediators of CACS.[5,6]

The area postrema (AP) is a sensory circumventricular organ of the caudal hindbrain, which is anatomically linked to and interacts in a reciprocal manner with the nucleus tractus solitarii (NTS).[7] Because of the lack of a functional blood-brain barrier, neurons of the AP represent targets for blood-borne hormones and pathological signalling molecules. The AP appears to be involved in pathological anorexia because lesions of the AP and the adjacent NTS region attenuate TNF-α-induced anorexia in rats.[8] Moreover, AP neurons are activated in tumour-bearing (TB) rats carrying hepatoma tumours.[9]

The NTS is an integrative relay centre for a variety of peripheral signals that control food intake.[10] It is the main projection site of vagal afferents from the gastrointestinal tract that transmits sensory visceral information to the brain. Vagotomy attenuated sickness-associated behaviours such as a decrease in social exploration induced by IL-1β or by the inflammatory endotoxin lipopolysaccharide (LPS).[11,12] On the other hand, subdiaphragmatic vagal deafferentation (SDA) failed to influence the anorectic effects of intraperitoneally injected LPS, muramyl dipeptide or IL-1β, indicating that vagal afferents are not necessary for the eating inhibition by these inflammatory mediators.[13,14] Experimental evidence for a role of the AP or vagal afferents in cancer anorexia is scarce. The only study addressing this question was conducted using a rat Leydig cell tumour model, which is characterized by endocrine activity of oestrogens.[15] Given that oestrogens act via the AP/NTS regions to reduce food intake,[16] the outcome of that study may reflect the consequences of hyperestrogenemia rather than specific tumour-dependent signalling mechanisms. Moreover, the lesioning approach in this study was not specific for the AP because the lesion included major parts of the NTS, resulting in a destruction of vagal terminal fields. Therefore, knowledge about the general role of the AP in cancer anorexia is very limited, and a better dissociation between vagal and AP-dependent mechanisms is required.

In the present study, we used neurosurgical approaches to examine the role of the AP and vagal afferents in the mediation of cancer anorexia and body weight loss in a rat hepatoma tumour model. A vacuum aspiration approach allowing the specific removal of the AP without compromising the structural integrity the NTS was used.[17] Moreover, we used the SDA technique, which is the most sophisticated deafferentation method because it disrupts all subdiaphragmatic vagal afferents while leaving about 50% of vagal efferents intact. The remaining vagal efferents are sufficient for maintaining the control of gastrointestinal function.[18] The comparison of APX and SDA approaches allows for the dissociation of vagal and AP mediated effects on CACS.

Despite their importance for disease-related anorexia, the classic pro-inflammatory cytokines including interleukins, TNF-α and IFN-γ do not seem to be required for the anorexia in the hepatoma tumour model.[9,19] There is cumulating evidence pointing to the AP as a target site for the signalling molecule MIC-1, also known as growth differentiation factor 15 (GDF15). Tumour cell dependent expression of MIC-1 can increase serum levels by up to 100-fold.[20–23] MIC-1 produced by xenografted tumours and recombinant protein both reduce food intake and lead to cachectic body weight loss in mice that can be inhibited by specific monoclonal antibodies.[24] Additionally, systemic injection of recombinant MIC-1 leads to rapid induction of c-Fos in the AP/NTS region, and a lesion of this brain area blocks the anorectic effect of exogenous MIC-1 in mice.[25] Given the recent evidence for a hindbrain-dependent suppression of food intake by MIC-1 and to further support the importance of MIC-1 signalling in CACS, we also sought to analyse MIC-1 blood levels at different time points during cancer progression.[25]

Materials and methods

Animals and housing conditions

Male Buffalo rats were used (Charles River Laboratory, USA). The animals were housed at controlled temperature (21 ± 1 °C) under a 12-h artificial light cycle with *ad libitum* access to standard laboratory rat chow (890 25 W16, Provimi Kliba, AG, Kaiseraugst, Switzerland). All experiments were approved by the Veterinary Office of the Canton Zurich.

Cell culture and tumour model

The hepatoma tumour model was described previously.[9,26] Morris hepatoma 7777 cells (McA-RH7777, Catalog No. CRL-1601, ATCC, USA) were cultured under standard conditions in DMEM supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin. Semi-confluent McA-RH7777 Petri dishes were washed with DMEM repeatedly to detach the cells from the surface. After confirming viability of the cells with trypan blue, 10^7 cells were inoculated subcutaneously between the scapulae in 250 μL PBS under short isoflurane anaesthesia. Control animals were also anaesthetized and injected with PBS.

Area postrema lesion

The APX was conducted as described.[27] Briefly, animals (200–230 g) were placed in a stereotactic frame with the head flexed ventrally in order to visualize the crista occipitalis. The atlanto-occipital membrane was dissected, and the meninges were carefully incised under surgical microscope control. The AP was then visualized and removed by vacuum aspiration using a blunted 26 G cannula connected to a water

vacuum pump. The animals were allowed 2 weeks for recovery before the start of the behavioural trials.

A histological verification of successful AP lesion was performed post-mortem. Microscopic coronal sections of the AP/NTS region were analysed. Only those animals in which the AP was removed without visible damage of the adjacent NTS were included in the study. Photomicrographs were taken at 20× magnification, using a Zeiss Imager Z2 microscope fitted with a digital camera system (Zeiss Axiocam).

Subdiaphragmatic vagal deafferentation

This surgical technique consists in a left-side intracranial transection of the vagal afferent rootlets and an ipsilateral transection of the dorsal subdiaphragmatic trunk of the vagus nerve resulting in complete SDA as previously described.[18] Rats (210–250 g) were pretreated with ip injections of 50 μg/kg atropine, antibiotics, and then anaesthetized with isoflurane. For sham-SDA surgery, the vagal rootlets and the dorsal subdiaphragmatic vagus trunk were exposed without disrupting them. Five millitre of warm saline and an analgesic were injected ip after suturing the abdomen. Post-surgical treatment with antibiotics and the analgesic continued for the following 2 days.

Two histological tests aimed to verify the completeness of SDA. These tests were based on published techniques for retrograde labelling of vagal motor neurons in the dorsal motor nucleus of the vagus (DMN) and anterograde labelling of vagal afferents in the NTS.[28–30] Rats were anaesthetized with isoflurane, and a midline incision at the level of the throat was made to expose the left nodose ganglion. A glass micropipette was inserted into the ganglion using a micromanipulator. A water solution (1.5 μL) containing the anterograde tracer biotinylated dextran amine (BDA, 5%) and 1% blue food colouring (Trawosa AG, St. Gallen, Switzerland) was pressure injected (PicoSpritzer 3; Parker Instrumentation, Fairfield, NJ) during 3–5 min. Three days after surgery the animals were shortly anaesthetized with isoflurane and 2 mg of the retrograde tracer fluorogold (FG) in 1 mL saline (Fluorochrome, Denver, CO) were injected intraperitoneally. After 2 days of tracer migration, the rats were perfused and the brains were removed as described below. The procedure for visualizing BDA-positive vagal projections in the NTS involved an incubation with the avidin-biotin-peroxidase complex followed by an incubation in 3,3′-diaminobenzidine solution (0.05% 3,3′-diaminobenzidine, 0.009% H_2O_2, 0.04% $NiCl_2$, 0.08% $CoCl_2$ in 0.05 M Tris-HCl). Absence of labelling was interpreted as successful transection of vagal afferents. To confirm completeness of unilateral subdiaphragmatic trunk vagotomy, an observer who was blind to the rat's surgery, counted the number of fluorogold-labelled neurons in the left and right DMN at the level of the AP. If the number of labelled cells found in the right DMN was less than 3% of the number found in the left DMN, and if vagal afferent fibres were absent in the NTS, the SDA surgery was considered successful. Sham-SDA rats were only included if FG-positive DMN neurons were labelled bilaterally and dense vagal afferent terminals were detected histologically. Photomicrographs were taken as described earlier.

Behavioural and metabolic studies

Using non-operated TB rats, we first sought to dissociate the effects of tumour anorexia on body weight and muscle loss from eating-independent effects. Non-tumour-bearing (NTB), pair-fed (PF), and TB rats (220–250 g) were single-housed in BIODAQ cages (Research Diets, NJ, USA) equipped with an external food hopper allowing precise 24-h food intake measurements. Rats were adapted to the housing conditions for 14 days before tumour cell injection. Daily food intake and body weight were measured shortly before dark-onset. Nine days after tumour induction, PF animals received the same amount of food as was consumed the previous day by the TB group. At the end of the experiment, the animals were euthanized for blood and tissue collection.

We then assessed the impact of tumour growth on metabolic rate and locomotor activity in this model. This experiment, which involved TB and NTB rats (130–150 g), was conducted in an open-circuit indirect calorimetric system (TSE Phenomaster, Bad Homburg, Germany) equipped with internal food hoppers and water bottles connected to scales, which allow for continuous recording of food and water consumption. The animals were single-housed in metabolic cages and adapted to the housing conditions for 7 days before the start of the experiment. Body weight was measured daily before dark onset. Food intake and respiratory gas exchange (O_2 and CO_2) were recorded automatically at 17-min intervals throughout the entire experiment. Locomotor activity (i.e. number of horizontal movements) was recorded using a frame of horizontal infrared beams (Accuscan, Columbus, Ohio).

Behavioural and metabolic studies with APX and SDA rats were also conducted in the same open-circuit indirect calorimetric system. In both studies, the animals were kept and adapted to the experimental conditions as described earlier. Food intake and body weight were recorded daily.

Terminal tissue and blood collection

Rats were anaesthetized shortly before dark onset after injection of pentobarbital (100 mg/kg ip). The thorax was opened and blood was collected from the right ventricle for subsequent MIC-1 measurements. Immediately afterwards, the rats were transcardially perfused with 0.1 M PB followed by 4% phosphate-buffered paraformaldehyde. The brains were

removed, post-fixed for 4 h in 4% phosphate-buffered paraformaldehyde and then cryoprotected for 2 days in 20% sucrose in 0.1 M PB at 4°C. Brains were subsequently frozen in hexane. Three series of coronal brainstem sections containing the AP/NTS region (20 μm) were cut with a cryomicrotome (CM3050S, Leica Microsystems, Germany), thaw-mounted on glass slides and then stored at −20°C until further processing.

MIC-1 measurements

Rats were single-housed in BIODAQ cages as described earlier. Food intake and body weight were measured daily and tumour growth was induced in all animals. Blood was obtained by puncturing the sublingual vein under mild isoflurane anaesthesia. Sampling was conducted 3 days before tumour induction (baseline) and 11 days and 17 days after induction, that is, shortly after the onset of anorexia and during fully developed anorexia, respectively. Blood was collected in EDTA containing tubes (Sarstedt, Nümbrecht, Germany) and centrifuged at 7000 × *g* (4°C, 7 min) to obtain plasma, which was stored in aliquots at −80°C for subsequent analysis. The levels MIC-1 were measured with using an ELISA (R&D Systems, USA) according to the manufacturers' instructions.

Body composition analysis and muscle and tumour weight measurements

After euthanizing the rats, tumours were resected and weighed. Total carcass lean and fat mass were measured by magnetic resonance imaging (EchoMRI, Echo Medical Systems, Texas, USA). Two consecutive measurements were taken to ensure instrument precision and averaged for the subsequent statistical analysis. The left gastrocnemius, tibialis, and soleus muscles were dissected at the level of their upper to lower tendons and weighed.

Data evaluation and statistical analysis

Mean daily food intake, body weight, body composition (lean and fat mass), and MIC-1 levels were expressed as mean ± SEM. In the pair-feeding experiment, body weight changes were calculated by subtracting the weight of the animal at the day of tumour induction from the final body weight. In the experiments involving AP-lesioned and vagotomized animals, food intake was corrected for body weight because at least in the APX study, the surgery had an effect on body weight. This effect is in line with previous studies in which APX animals tended to gain less weight than sham-APX because of moderately decreased total food intake.[17] Weekly body weight change after tumour-induction was calculated by subtracting the body weight at the beginning of each week from the body weight at the end of same week. Food intake and body weight changes were quantified across a 3-week period starting one week before the onset of anorexia. Presumably, because of general biological variability, anorexia in the APX study started a week later than in the SDA study, that is, 3 instead of 2 weeks after tumour induction. Metabolic rate was calculated from O_2 consumption and CO_2 production as described previously.[31] Metabolic rate data were normalized for body weight. The calculation was based on the following equation: total EE (kcal/kg/h) = $(3.9 \times VO_2 + 1.1 \times VCO_2) / 1000$. The average values obtained between Days 1 and 3 after tumour inoculation were used as baseline (i.e. prior to the onset of anorexia) and compared with the average values during three consecutive days in each week after tumour induction. The number of horizontal movements (i.e. locomotor activity) was monitored across continuous 5-min intervals over 24 h and expressed as average of three consecutive days.

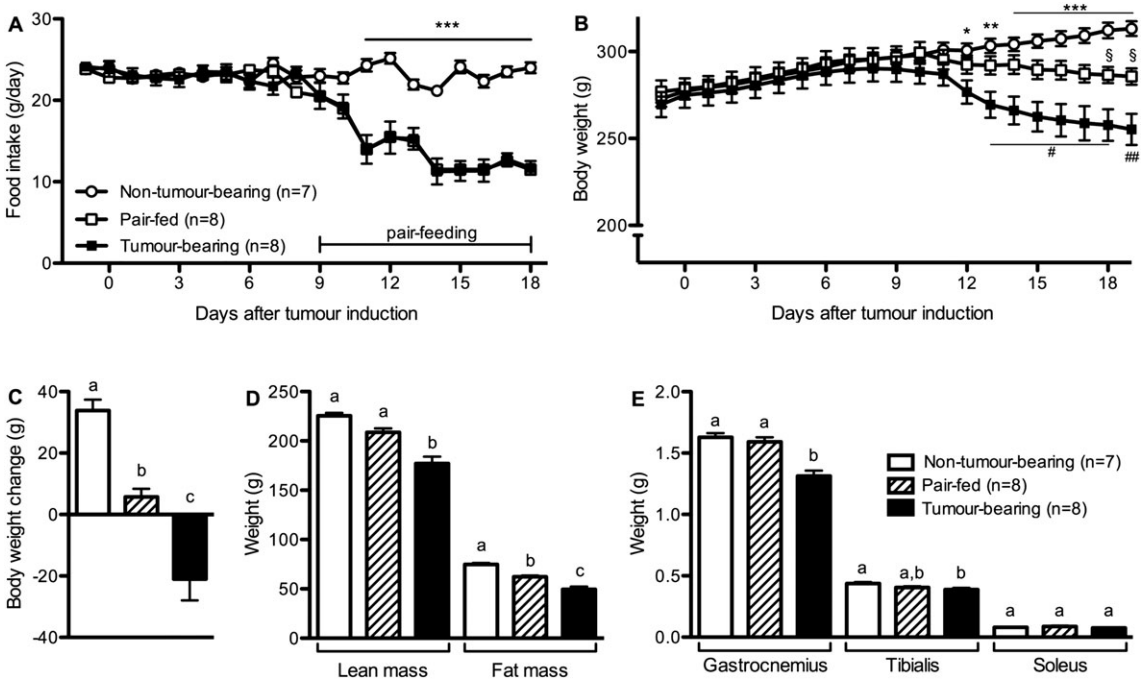
Statistical comparisons between multiple groups were performed using one-way ANOVA followed by Tukey's post-hoc test. Unpaired Student's *t*-test (two-sided) was used for comparison between two groups. Linear regression analysis was used to examine the correlation between MIC-1 levels, tumour weight and food intake. For all statistical tests, a *P*-value less than 0.05 was considered significant. Data were analysed using Prism GraphPad 5.0.

Results

Effects of tumour growth on food intake, body weight and muscle, fat and lean mass

Tumours became palpable between Days 9 and 11, which coincided with the onset of the anorectic response. TB rats showed significantly lower food intake than NTB animals from Day 11 after tumour cell inoculation. Daily food intake stabilized at a lower level with an average reduction of 45% in comparison to NTB animals (*Figure* 1A). While NTB animals displayed constant body weight gain during the experimental period, TB animals showed a marked loss of body weight, which became significant 12 days after tumour induction. Pair-feeding reduced body weight gain, which led to a significantly lower body weight compared with NTB on Days 18–19. Pair-feeding did not, however, lower body weight to the level of the TB animals; these animals were significantly heavier than the TB rats on Day 13 (*Figure* 1B–C). TB animals had lower lean carcass mass in comparison with healthy controls and PF animals (*Figure* 1D). TB rats also had lower fat mass than controls and PF rats. Furthermore, TB rats had lower gastrocnemius and tibialis muscle mass than NTB animals (*Figure* 1E), while muscle weight of the PF group did not differ from NTB controls. The tumour weight was 13.2 ± 1.5 g at the end of experiment.

Figure 1 Tumour-induced body weight loss and muscle degradation are partly independent of anorexia. (A–C) Tumour-bearing rats developed anorexia and lost body weight. Pair-feeding only led to an attenuation of body weight gain that was calculated by subtracting the body weight at the time of tumour induction from the body weight at the end of the experiment. (D) Tumour-bearing animals had lower lean and fat carcass mass compared with both control groups. (E) Tumour-bearing rats had lower gastrocnemius and tibialis muscle mass compared with Non-Tumour-bearing animals and lower gastrocnemius muscle mass compared with the pair-fed group. Data analysed with Student's *t*-test (A) or with one-way ANOVA followed by Tukey's post-hoc test (B–E). Means with different letter or symbols are significantly different from each other; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, same for # and §).



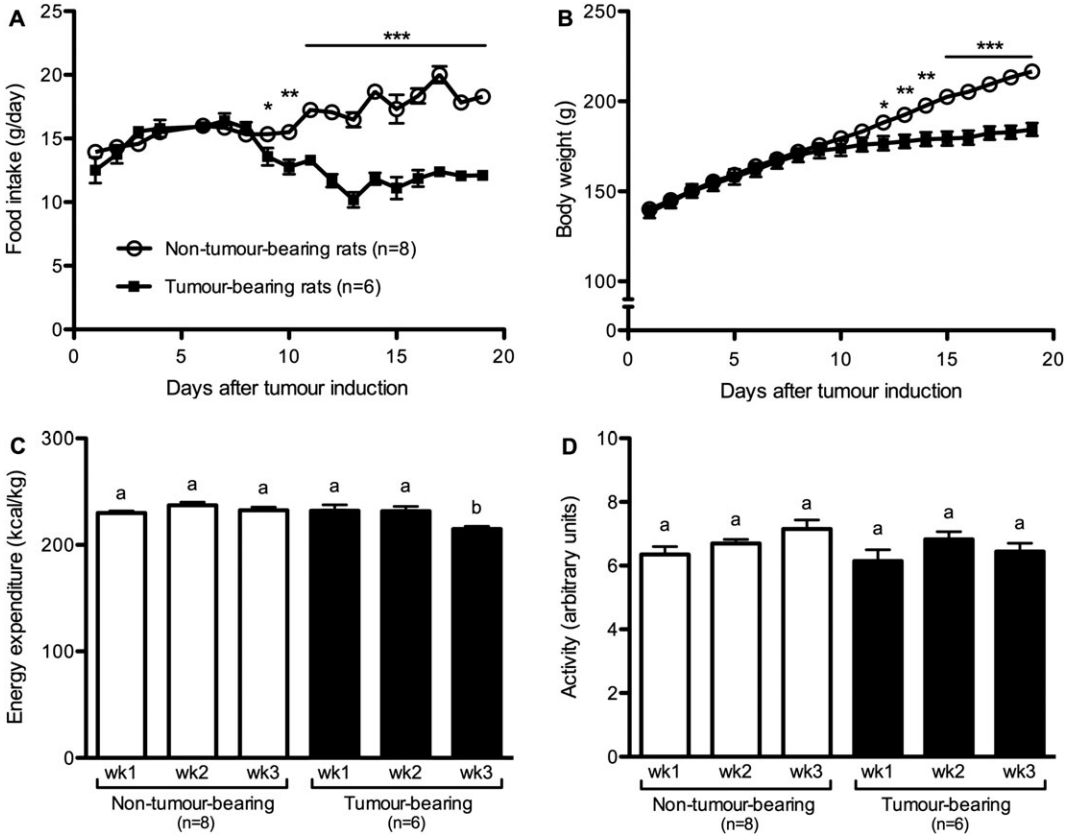
Effects of tumour growth on metabolism and locomotor activity

Energy expenditure and activity were analysed in a separate cohort of TB and NTB rats. The food intake of these animals following tumour induction decreased, leading to significantly lower body weight in TB rats compared to NTB controls (*Figure* 2A–B). While the metabolic rate of the NTB controls did not significantly change during the experiment, TB animals showed a slight but significant reduction in metabolic rate during the third week after tumour induction (*Figure* 2C). Daily locomotor activity was similar between the two experimental groups (*Figure* 2D) and there were no differences in the diurnal and nocturnal activity pattern (data not shown).

Area postrema lesion blocked anorexia and attenuated tumour-induced body weight loss and muscle degradation

Body weight before tumour induction was lower in the APX animals compared with sham-APX rats (APX: 225 ± 9 vs. sham-APX 262 ± 8.5 g). However, no significant differences in food intake between the experimental groups occurred prior to tumour anorexia when food intake was corrected for body weight (APX: 7.2 ± 0.2 vs. sham-APX: 7.6 ± 0.2 g/100 g BW). The food intake of the sham-APX rats decreased following tumour induction, resulting in a significant reduction of $40 \pm 3\%$ between Weeks 2 and 4 (*Figure* 3A). This effect was accompanied by a decline in body weight gain, leading to net body weight loss in Week 4 (*Figure* 3B). Strikingly, and in contrast to its effect in sham-APX rats, tumour growth did not affect food intake in APX animals (*Figure* 3A). The weekly body weight gain of the APX rats only significantly decreased in Week 4. In contrast to sham-APX rats, however, APX animals did not lose body weight following tumour growth (*Figure* 3B). Furthermore, APX rats had higher gastrocnemius and soleus muscle weights than controls (*Figure* 3C). The metabolic rate was similar between APX and sham-APX animals. Tumour growth decreased metabolic rate in both surgical groups similarly with a significant reduction between Weeks 2 and 3. No further decrease occurred between Weeks 3 and 4 (APX vs. sham-APX, wk2: 185.8 ± 10.3 vs. 186.1 ± 5.4; wk3: 160.6 ± 2.6, 160.0 ± 1.3; wk4: 162.2 ± 4.8 vs. 159.6 ± 3.4 kcal/kg/d). Locomotor activity was similar in APX and sham-APX animals and no significant changes occurred between the two groups following tumour induction (APX vs. sham-APX, wk2: 5.5 ± 0.5 vs. 5.6 ± 0.4; wk3: 6.6 ± 0.4 vs.

Figure 2 Tumour growth reduced metabolic rate without affecting locomotor activity. Tumour-bearing rats developed anorexia (A) and showed reduced body weight gain (B), but did not show differences in metabolic rate (C) or locomotor activity (D), except during Week 3 in which tumour-bearing rats showed reduced metabolic rate. Data analysed with Student's *t*-test (A–B), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data analysed with one-way ANOVA followed by Tukey's post-hoc test (C–D). Means with different letters are significantly different from each other ($P < 0.05$).



6.7 ± 0.5; wk4: 6.0 ± 0.5 vs. 6.7 ± 0.3 arbitrary units). Importantly, tumour weight did not differ significantly between APX and sham-APX rats at the end of the experiment (9.6 ± 0.7 vs. 7.7 ± 2.3 g). Representative histological sections of the AP/NTS region from a sham-APX and an APX animal are shown in *Figure* 3D.

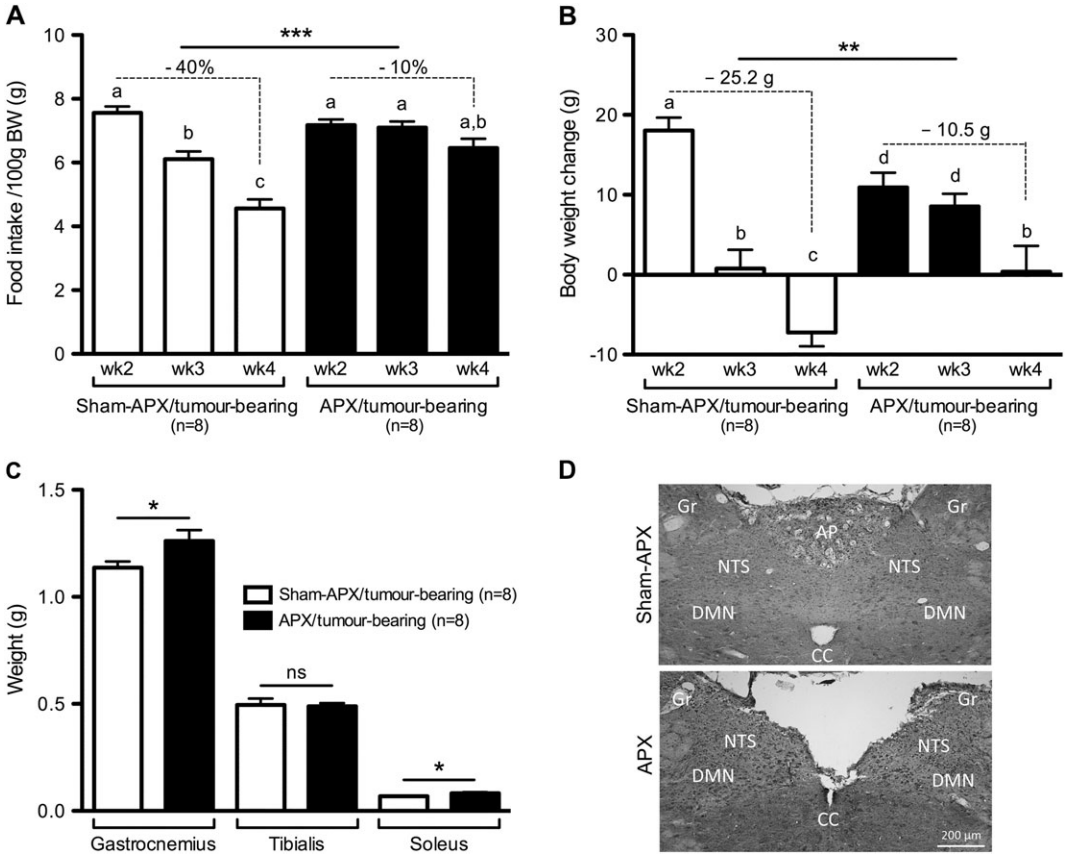
Subdiaphragmatic vagal deafferentation did not prevent tumour-induced anorexia and body weight loss

In line with previous reports, daily food intake did not differ between SDA and sham-SDA animals prior to tumour induction (21.8 ± 0.9 SDA vs. 22.3 ± 0.5 g sham-SDA).[32,33] Moreover, body weight did not differ between the two groups before the surgery (SDA: 253 ± 4 vs. sham-SDA: 246 ± 3 g) and prior to tumour induction (SDA: 252 ± 5 vs. sham-SDA: 248 ± 4 g). In contrast to APX, SDA did not affect tumour-induced anorexia. Tumour growth reduced food intake in SDA and sham-SDA rats similarly, leading to a food intake reduction of 39 ± 3 and 41 ± 2% between Weeks 1 and 3 in SDA and sham-SDA rats, respectively (*Figure* 4A). Likewise, tumour growth attenuated body weight gain in both groups during Week 2 similarly, resulting in body weight loss in Week 3 (*Figure* 4B). No differences in lean and fat mass, metabolic rate, and locomotor activity were observed between the two groups (data not shown). The tumour weight of the SDA and sham-SDA animals did not differ at the end of the experiment (17.6 ± 2.6 vs. 21.8 ± 1.9 g). *Figure* 4C shows representative histological sections of animals in both groups. Unilateral anterograde labelling of vagal afferents in the NTS was visible in sham-SDA but absent in SDA rats. Furthermore, sham-SDA rats showed bilateral retrograde labelling of vagal motor neurons in the DMN, whereas only unilateral staining of the DMN was present in SDA animals.

Tumour growth and anorexia correlated with MIC-1 blood levels

MIC-1 plasma levels in TB rats were significantly higher than in NTB and PF controls at the end of the experiment, showing a 42-fold increase (*Figure* 5A). MIC-1 levels did not differ between PF and NTB rats. In a separate group of animals, MIC-1

Figure 3 Lesion of the area postrema attenuated anorexia, body weight loss and muscle degradation induced by tumour growth. (A–B) Area postrema lesioned (APX) animals were protected against tumour-induced anorexia and showed markedly attenuated body weight loss following tumour induction. (C) Area postrema lesioned rats show higher gastrocnemius and soleus mass compared to area postrema-sham lesioned (sham-APX) tumour-bearing animals. (D) Coronal sections of the area postrema/nucleus tractus solitarii of a sham-lesioned control (upper image) and an area postrema lesioned animal (lower image). AP, area postrema; NTS, nucleus tractus solitarii; DMN, dorsal motor nucleus of the vagus; Gr, gracile nucleus; CC, central canal. Data analysed with one-way ANOVA followed by Tukey's post-hoc test (A–B), means with different letters are significantly different from each other ($P < 0.05$). Changes in food intake, differences in body weight change between Weeks 2 and 4, and muscle weights were analysed using the Student's *t*-test (A-C), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.



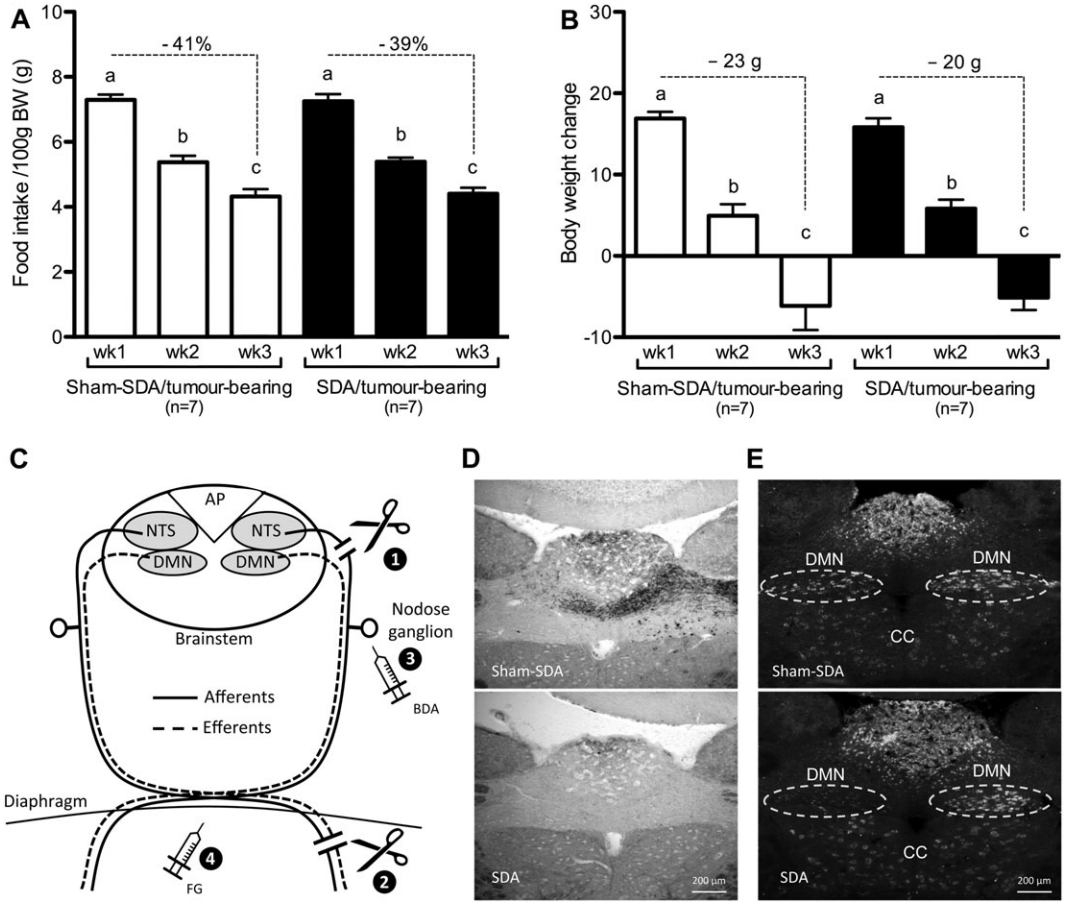
levels were also measured before tumour induction, shortly after the onset of anorexia and at the end of the experiment (11 and 17 days after tumour induction, respectively). MIC-1 plasma levels were already elevated 11 days after tumour induction compared with baseline conditions and increased further until Day 17 (*Figure* 5B). MIC-1 levels positively correlated with tumour size (*Figure* 5C) and with the severity of anorexia during tumour growth (*Figure* 5D).

Discussion

The role of the AP and of vagal afferents in chronic cancer anorexia and body weight loss has neither been critically examined nor conclusively dissociated. Using the specific APX and SDA approaches, we now provide evidence that the AP is required for cancer anorexia and body weight loss in hepatoma TB rats. Lesion of the AP also attenuated cancer-dependent muscle loss. In contrast to APX, SDA did not affect cancer-induced anorexia and body weight loss, indicating that vagal afferents are not necessary for the mediation of CACS in this model. We also identified MIC-1 as a possible mediator of CACS in hepatoma TB rats because MIC-1 levels correlated with tumour progression and tumour size, and correlated negatively with food intake.

As demonstrated in previous studies, hepatoma TB rats showed an activation of the AP/NTS region that was caused by tumour growth but not as a consequence of anorexia because it did not occur in pair-fed NTB controls.[9] Our studies extend this observation by demonstrating clear differences between TB and pair-fed NTB rats with respect to body weight, body composition, and muscle mass. TB rats lost considerably more body weight than NTB pair-fed rats and showed a reduction in total lean mass and hind limb muscle mass. These findings are consistent with the notion that

Figure 4 Subdiaphragmatic vagal deafferentation did not attenuate cancer-anorexia-cachexia syndrome. (A) Tumour growth induced a strong anorectic response in both subdiaphragmatic vagal deafferentation (SDA) and sham-operated (sham-SDA) animals. (B) In both groups anorexia was paralleled by a similar reduction of body weight gain in Week 2 and a net body weight loss in Week 3. (C) Schematic illustration of afferent and efferent vagal fibres targeted by the subdiaphragmatic vagal deafferentation and of the procedures used to verify its completeness. The subdiaphragmatic vagal deafferentation consists in a left intracranial rhizotomy of all dorsal vagal fibres (i.e. afferent) (1) and a complete subdiaphragmatic dissection (afferent and efferent fibers) of the left trunk of the vagus nerve (2). With this surgical procedure all vagal afferents are dissected, leaving 50% of the vagal efferents intact. Biotinylated dextran amine (BDA) was applied directly into the nodose ganglion of the vagus nerve 5 days prior to sacrifice (3). Fluorogold (FG) was injected intraperitoneally 48 h prior to sacrifice (4). D–E) Coronal sections of the area postrema/nucleus tractus solitarii region of a sham-lesioned control and a subdiaphragmatic vagal deafferentation animal. (D) While biotinylated dextran amine-positive fibers were present in the nucleus tractus solitarii of sham rats, no labelling was observed in the nucleus tractus solitarii of subdiaphragmatic vagal deafferentation animals. (E) While bilateral Fluorogold staining in the dorsal motor nucleus (DMN) of sham rats was observed, only unilateral staining of the dorsal motor nucleus occurred in subdiaphragmatic vagal deafferentation animals. CC, central canal. One-way ANOVA followed by Tukey's post-hoc test, means with different letters are significantly different from each other ($P < 0.05$).

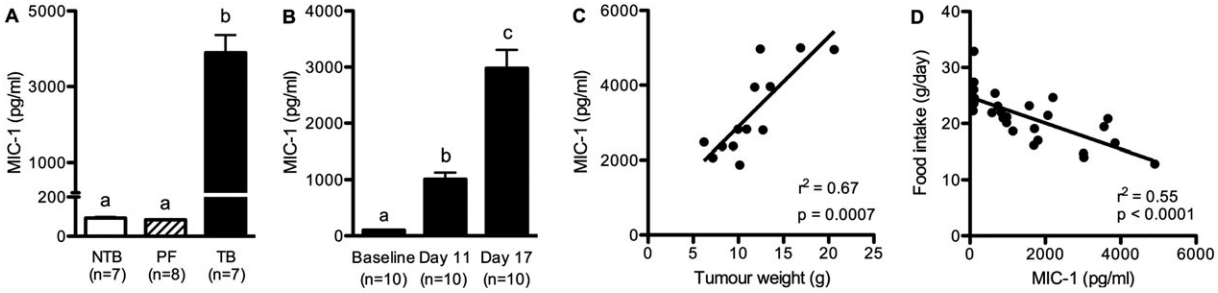


tumour-dependent body weight loss and muscle degradation is not only due to reduced energy intake.

Differences in metabolic rate and locomotor activity between TB and NTB were small or absent, respectively. The only significant difference consisted of a reduced metabolic rate of TB rats during Week 3 after tumour induction. The consequences of cancer growth for energy expenditure vary among tumour models depending on the status of inflammation and effects caused by changes in food intake or physical activity.[34,35] In our studies, the moderately reduced metabolic rate of TB rats might be related to the reduced food intake.

Notably, indirect calorimetry does not reflect possible changes in energy expenditure because of anaerobic metabolism, which particularly occurs in tumour tissues, including the hepatoma cells used in these studies.[36,37] The considerably greater body weight loss in TB vs. pair-fed NTB rats in the absence of marked differences in aerobic metabolic rate and locomotor activity suggests a role of anaerobic hypermetabolism in this tumour model. Another possible reason for the difference in body weight between TB and PF animals could be related to a maladaptation of energy expenditure, following reduced caloric intake. Reduction in metabolic rate during food-restriction is a well-known adaptive mechanism

Figure 5 Macrophage inhibitory cytokine-1 levels increased with tumour progression and correlated with tumour size and the severity of anorexia (A) macrophage inhibitory cytokine-1 plasma levels were significantly higher in tumour-bearing rats compared to controls at the end of the experiment. (B) macrophage inhibitory cytokine-1 levels were elevated 11 days after tumour induction (i.e. 3 days after anorexia onset) and further increased with cancer progression. (C) Macrophage inhibitory cytokine-1 levels correlated with tumour weight. (D) Macrophage inhibitory cytokine-1 levels negatively correlated with food intake during tumour growth. Data analysed with one-way ANOVA followed by Tukey's post-hoc test (A–B), means with different letters are significantly different from each other ($P < 0.05$).



preventing excessive body weight loss. While this mechanism might have partially protected PF animals from body weight loss, an adaptive reduction in metabolic rate might have been negligible in TB rats.[38,39] We did not directly compare metabolic rate of PF and TB rats, but the small reduction in metabolic rate of TB rats compared with NTB animals is in line with this assumption.

In contrast to earlier studies using AP/NTS lesions, the NTS was not lesioned in our study.[15] This is important because it means that the neuroanatomy conveying vagal afferent signalling to the hindbrain was still intact in our APX study. Furthermore, by using SDA, we minimized the severe impairment of gastrointestinal function that usually occurs in other experimental approaches (e.g. complete subdiaphragmatic vagotomy). Although lesion of the AP moderately reduces food intake and body weight compared with controls,[17,27] APX animals are still sensitive to anorectic stimuli such as LPS or CCK.[40,41] Therefore, lesion of the AP does not create a state of general unresponsiveness to anorectic stimuli. While APX rats did not develop cancer anorexia or body weight loss, body weight gain significantly decreased in the last week of the experiments. As discussed earlier, anaerobic hypermetabolism might have contributed to reduced body weight gain. Furthermore, mean daily food intake tended to decrease during the last week leading to a non-significant reduction of cumulative food intake by about 10 g compared with the week before. We did not attempt to confirm or exclude the possible reasons for the reduced body weight gain toward the end of the experiment because the different effects mentioned earlier are likely to act in concert.

Most importantly, however, our studies clearly demonstrate an amelioration of CACS in APX rats including an attenuation of muscle loss. Differences in tumour growth did not account for decreased loss of muscle mass, because APX did not affect the tumour size. The lesion of the AP *per se* is also unlikely to increase muscle mass because APX rats generally tend to have lower body weights compared with sham-APX rats. The pathological mechanisms leading to muscle wasting in the present tumour model have not yet been explored. In general, pro-inflammatory cytokines are important mediators of cancer-dependent muscle wasting acting via muscle degradation pathways.[42] So far, no increased levels of TNF-α, IL-1β, IL-6, or IFN-γ have been identified in this tumour model.[9] TB APX rats might be able to partially preserve muscle mass because of higher energy intake than TB sham-APX rats, but pair-feeding experiments would be required to confirm this.

The persistence of CACS in SDA rats may seem surprising given the presumed role of vagal afferents for the transmission of sickness-related signals to the brain.[43] While the SDA approach is the most appropriate surgical technique to disrupt vagal afferent signalling, we cannot exclude that afferent signals originating from the right nodose ganglion could be transmitted to the brainstem. However, the effects of anorectic stimuli such as CCK are blocked by SDA, suggesting that an intact nodose ganglion is not sufficient for the suppression of food intake induced by vagus-dependent anorectic stimuli.[44] Based on almost identical anorexia and body weight loss in SDA compared with sham-SDA animals, it appears unlikely that the full CACS response is mediated via a unilateral supradiaphragmatic vagal mechanism.

Whereas in some studies, complete subdiaphragmatic vagotomy has been shown to reduce different sickness-related symptoms including LPS and IL-1β-induced anorexia,[45] the more specific SDA procedure that we employed here did not attenuate the anorectic response to immunomodulators.[13,14] Moreover, all these studies investigated the involvement of the vagus nerve in sickness anorexia accompanying acute models of inflammation, for example, treatment with endotoxins such as LPS or muramyl dipeptide or acute injection of pro-inflammatory cytokines. To our knowledge, the only study exploring the role of the vagus in cancer anorexia was conducted in Leydig cell TB rats. Both total vagotomy and capsaicin-induced vagal damage attenuated tumour anorexia in this tumour model.[46] Whether the vagal mediation

of cancer anorexia in this model is related to oestrogen produced by the tumour remains unclear but represents a plausible explanation.

Overall, the heterogeneity of findings regarding the involvement of vagal afferent signalling in disease-related anorexia may reflect differences in pathological characteristics of the pertinent disease models. Although we cannot generalize our finding that vagal afferents did not contribute to cancer anorexia, the clear-cut dissociation of vagal vs. AP-dependent mechanisms helps to narrow down possible pathomechanisms and therapeutic targets.

There is much evidence for the pivotal role of pro-inflammatory cytokines in the development of CACS.[47] In contrast to other tumour models,[48] no increases in circulating cytokines such as IL-1β, IFN-γ, IL-6, and TNF-α have been observed in hepatoma TB rats.[9] We therefore hypothesized that other cytokines or tumour-derived factors might contribute to CACS in this tumour model. Consistent with this hypothesis, we identified clearly elevated levels of MIC-1 in TB rats, which positively correlated with tumour size and anorexia. The increase in circulating MIC-1 was not secondary to anorexia because pair-fed NTB rats had basal MIC-1 levels comparable with *ad libitum* fed controls. This finding parallels recent observations in humans demonstrating that MIC-1 levels do not seem to be determined by the amount of food intake.[49] Different types of tumours express high levels of MIC-1.[21–23] Under inflammatory conditions, MIC-1 can also be expressed by host tissue.[50] While we have not identified the origin of MIC-1 in TB rats, the lack of a general elevation in pro-inflammatory cytokines and the strong correlation between tumour size and MIC-1 levels suggest the tumour as a likely source of MIC-1 in hepatoma TB rats.

Our studies complement previous findings suggesting that MIC-1 contributes to CACS via an AP/NTS-dependent action as MIC-1-induced anorexia and body weight loss was attenuated in mice with AP/NTS lesion.[25] While only a combined AP/NTS lesion completely blunted the effect of MIC-1 treatment on body weight, mice with specific lesions of the AP showed reduced body weight gain but were protected against body weight loss.

The clinical relevance of MIC-1 as a possible mediator of CACS in humans has primarily been evaluated based on correlations of MIC-1 blood levels with tumour and disease parameters (e.g. body weight loss). While in many clinical studies MIC-1 levels correlated with the severity of body weight loss in cancer patients,[24,51] other studies did not observe such a correlation.[52,53] Several clinical studies highlighted the potential use of MIC-1 as a biological marker for prognosis and diagnosis of certain types of cancer[52,54,55] (see Fairlie *et al*.[56] for review). It is important to highlight that the clinical relevance of MIC-1 in human cancer patients appears to vary depending on the type of cancer.

Collectively, our studies substantiate the role of the AP in CACS. We also identified MIC-1 as a possible humoral mediator of anorexia and body weight loss in hepatoma TB rats. Both MIC-1 and local signalling processes in the AP/NTS region (e.g. GLP-1) represent promising therapeutic targets for the treatment of CACS.

Acknowledgement

The authors certify that they comply with the ethical guidelines for authorship and publishing of the Journal of Cachexia, Sarcopenia, and Muscle update 2015.[57]

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Conflict of interest

None declared.

References

1. Evans WJ, Morley JE, Argiles J, Bales C, Baracos V, Guttridge D, et al. Cachexia: a new definition. *Clin Nutr* 2008;**27**:793–799.
2. Tisdale MJ. Biology of cachexia. *J Natl Cancer Inst* 1997;**89**:1763–1773.
3. Gordon JN, Green SR, Goggin PM. Cancer cachexia. *QJM* 2005;**98**:779–788.
4. Tisdale MJ. Cachexia in cancer patients. *Nat Rev Cancer* 2002;**2**:862–871.
5. Plata-Salaman CR. Cytokines and feeding. *Int J Obes Relat Metab Disord* 2001;**25**: S48–552.
6. Laviano A, Meguid MM, Rossi-Fanelli F. Cancer anorexia: clinical implications, pathogenesis, and therapeutic strategies. *Lancet Oncol* 2003;**4**:686–694.
7. Vigier D, Rouviere A. Afferent and efferent connections of the area postrema demonstrated by the horseradish peroxidase method. *Arch Ital Biol* 1979;**117**:325–339.
8. Bernstein IL, Taylor EM, Bentson KL. TNF-induced anorexia and learned food aversions are attenuated by area postrema lesions. *Am J Physiol* 1991;**260**:R906–R910.
9. Ruud J, Blomqvist A. Identification of rat brainstem neuronal structures activated during cancer-induced anorexia. *J Comp Neurol* 2007;**504**:275–286.
10. Grill HJ, Hayes MR. The nucleus tractus solitarius: a portal for visceral afferent signal processing, energy status assessment and integration of their combined effects on food intake. *Int J Obes (Lond)* 2009;**33**: S11–S15.
11. Bluthe RM, Michaud B, Kelley KW, Dantzer R. Vagotomy attenuates behavioural effects of interleukin-1 injected peripherally but not centrally. *Neuroreport* 1996;**7**:1485–1488.
12. Bluthe RM, Walter V, Parnet P, Laye S, Lestage J, Verrier D, et al. Lipopolysaccharide induces sickness behaviour in rats by a vagal mediated mechanism. *C R Acad Sci III* 1994;**317**:499–503.
13. Porter MH, Hrupka BJ, Langhans W, Schwartz GJ. Vagal and splanchnic afferents are not necessary for the anorexia produced by peripheral IL-1beta, LPS, and MDP. *Am J Physiol* 1998;**275**:R384–R389.

14. Schwartz GJ, Plata-Salaman CR, Langhans W. Subdiaphragmatic vagal deafferentation fails to block feeding-suppressive effects of LPS and IL-1 beta in rats. *Am J Physiol* 1997;**273**:R1193–R1198.
15. Bernstein IL, Treneer CM, Kott JN. Area postrema mediates c effects on food intake, body weight, and learned aversions. *Am J Physiol* 1985;**249**:R296–R300.
16. Asarian L, Geary N. Sex differences in the physiology of eating. *Am J Physiol Regul Integr Comp Physiol* 2013;**305**:R1215–R1267.
17. Lutz TA, Senn M, Althaus J, Del Prete E, Ehrensperger F, Scharrer E. Lesion of the area postrema/nucleus of the solitary tract (AP/NTS) attenuates the anorectic effects of amylin and calcitonin gene-related peptide (CGRP) in rats. *Peptides* 1998;**19**:309–317.
18. Norgren R, Smith GP. A method for selective section of vagal afferent or efferent axons in the rat. *Am J Physiol* 1994;**267**: R1136–R1141.
19. Langhans W, Hrupka B. Interleukins and tumor necrosis factor as inhibitors of food intake. *Neuropeptides* 1999;**33**:415–424.
20. Bauskin AR, Brown DA, Kuffner T, Johnen H, Luo XW, Hunter M, et al. Role of macrophage inhibitory cytokine-1 in tumorigenesis and diagnosis of cancer. *Cancer Res* 2006;**66**:4983–4986.
21. Staff AC, Bock AJ, Becker C, Kempf T, Wollert KC, Davidson B. Growth differentiation factor-15 as a prognostic biomarker in ovarian cancer. *Gynecol Oncol* 2010;**118**:237–243.
22. Welsh JB, Sapinoso LM, Kern SG, Brown DA, Liu T, Bauskin AR, et al. Large-scale delineation of secreted protein biomarkers overexpressed in cancer tissue and serum. *Proc Natl Acad Sci U S A* 2003;**100**:3410–3415.
23. Zimmers TA, Jin X, Gutierrez JC, Acosta C, McKillop IH, Pierce RH, et al. Effect of *in vivo* loss of GDF-15 on hepatocellular carcinogenesis. *J Cancer Res Clin Oncol* 2008;**134**:753–759.
24. Johnen H, Lin S, Kuffner T, Brown DA, Tsai VW, Bauskin AR, et al. Tumor-induced anorexia and weight loss are mediated by the TGF-beta superfamily cytokine MIC-1. *Nat Med* 2007;**13**:1333–1340.
25. Tsai VW, Manandhar R, Jorgensen SB, Lee-Ng KK, Zhang HP, Marquis CP, et al. The anorectic actions of the TGFbeta cytokine MIC-1/GDF15 require an intact brainstem area postrema and nucleus of the solitary tract. *PLoS One* 2014;**9**:e100370.
26. Borner T, Loi L, Pietra C, Giuliano C, Lutz TA, Riediger T. The ghrelin receptor agonist HM01 mimics the neuronal effects of ghrelin in the arcuate nucleus and attenuates anorexia-cachexia syndrome in tumor-bearing rats. *Am J Physiol Regul Integr Comp Physiol* 2016.
27. Braegger FE, Asarian L, Dahl K, Lutz TA, Boyle CN. The role of the area postrema in the anorectic effects of amylin and salmon calcitonin: behavioral and neuronal phenotyping. *Eur J Neurosci* 2014;**40**:3055–3066.
28. Powley TL, Fox EA, Berthoud HR. Retrograde tracer technique for assessment of selective and total subdiaphragmatic vagotomies. *Am J Physiol* 1987;**253**: R361–R370.
29. Walls EK, Wang FB, Holst MC, Phillips RJ, Voreis JS, Perkins AR, et al. Selective vagal rhizotomies: a new dorsal surgical approach used for intestinal deafferentations. *Am J Physiol* 1995;**269**:R1279–R1288.
30. Norgren R, Smith GP. Central distribution of subdiaphragmatic vagal branches in the rat. *J Comp Neurol* 1988;**273**:207–223.
31. Riediger T, Cordani C, Potes CS, Lutz TA. Involvement of nitric oxide in lipopolysaccharide induced anorexia. *Pharmacol Biochem Behav* 2010;**97**:112–120.
32. Ferrari B, Arnold M, Carr RD, Langhans W, Pacini G, Bodvarsdottir TB, et al. Subdiaphragmatic vagal deafferentation affects body weight gain and glucose metabolism in obese male Zucker (fa/fa) rats. *Am J Physiol Regul Integr Comp Physiol* 2005;**289**:R1027–R1034.
33. Arnold M, Mura A, Langhans W, Geary N. Gut vagal afferents are not necessary for the eating-stimulatory effect of intraperitoneally injected ghrelin in the rat. *J Neurosci* 2006;**26**:11052–11060.
34. Tisdale MJ. Mechanisms of cancer cachexia. *Physiol Rev* 2009;**89**:381–410.
35. Bennani-Baiti N, Walsh D. Animal models of the cancer anorexia-cachexia syndrome. *Support Care Cancer* 2011;**19**:1451–1463.
36. Mares-Perlman JA, Shrago E. Energy substrate utilization in freshly isolated Morris Hepatoma 7777 cells. *Cancer Res* 1988;**48**:602–608.
37. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* 2009;**324**:1029–1033.
38. Tisdale MJ. Metabolic abnormalities in cachexia and anorexia. *Nutrition* 2000;**16**:1013–1014.
39. Ihnatko R, Post C, Blomqvist A. Proteomic profiling of the hypothalamus in a mouse model of cancer-induced anorexia-cachexia. *Br J Cancer* 2013;**109**:1867–1875.
40. Weingarten S, Senn M, Langhans W. Does a learned taste aversion contribute to the anorectic effect of bacterial lipopolysaccharide? *Physiol Behav* 1993;**54**:961–966.
41. Edwards GL, Ladenheim EE, Ritter RC. Dorsomedial hindbrain participation in cholecystokinin-induced satiety. *Am J Physiol* 1986;**251**:R971–R977.
42. Burckart K, Beca S, Urban RJ, Sheffield-Moore M. Pathogenesis of muscle wasting in cancer cachexia: targeted anabolic and anticatabolic therapies. *Curr Opin Clin Nutr Met Care* 2010;**13**:410–416.
43. McCarthy DO. Cytokines and the anorexia of infection: potential mechanisms and treatments. *Biol Res Nurs* 2000;**1**:287–298.
44. Moran TH, Baldessarini AR, Salorio CF, Lowery T, Schwartz GJ. Vagal afferent and efferent contributions to the inhibition of food intake by cholecystokinin. *Am J Physiol* 1997;**272**:R1245–R1251.
45. Maier SF, Goehler LE, Fleshner M, Watkins LR. The role of the vagus nerve in cytokine-to-brain communication. *Ann N Y Acad Sci* 1998;**840**:289–300.
46. Bernstein IL. Neutral mediation of food aversions and anorexia induced by tumor necrosis factor and tumors. *Neurosci Biobehav Rev* 1996;**20**:177–181.
47. Laviano A, Meguid MM, Inui A, Muscaritoli M, Rossi-Fanelli F. Therapy insight: cancer anorexia-cachexia syndrome--when all you can eat is yourself. *Nat Clin Pract Oncol* 2005;**2**:158–165.
48. Talbert EE, Metzger GA, He WA, Guttridge DC. Modeling human cancer cachexia in colon 26 tumor-bearing adult mice. *J Cachexia Sarcopenia Muscle* 2014;**5**:321–328.
49. Tsai VW, Macia L, Feinle-Bisset C, Manandhar R, Astrup A, Raben A, et al. Serum levels of human MIC-1/GDF15 vary in a diurnal pattern, do not display a profile suggestive of a satiety factor and are related to BMI. *PLoS One* 2015;**10**:e0133362.
50. Fairlie WD, Moore AG, Bauskin AR, Russell PK, Zhang HP, Breit SN. MIC-1 is a novel TGF-beta superfamily cytokine associated with macrophage activation. *J Leukoc Biol* 1999;**65**:2–5.
51. Lerner L, Hayes TG, Tao N, Krieger B, Feng B, Wu Z, et al. Plasma growth differentiation factor 15 is associated with weight loss and mortality in cancer patients. *J Cachexia Sarcopenia Muscle* 2015;**6**:317–324.
52. Skipworth RJ, Deans DA, Tan BH, Sangster K, Paterson-Brown S, Brown DA, et al. Plasma MIC-1 correlates with systemic inflammation but is not an independent determinant of nutritional status or survival in oesophago-gastric cancer. *Br J Cancer* 2010;**102**:665–672.
53. Lu Z, Yang L, Yu J, Lu M, Zhang X, Li J, et al. Change of body weight and macrophage inhibitory cytokine-1 during chemotherapy in advanced gastric cancer: what is their clinical significance? *PLoS One* 2014;**9**: e88553.
54. Brown DA, Ward RL, Buckhaults P, Liu T, Romans KE, Hawkins NJ, et al. MIC-1 serum level and genotype: associations with progress and prognosis of colorectal carcinoma. *Clin Cancer Res* 2003;**9**:2642–2650.
55. Koopmann J, Buckhaults P, Brown DA, Zahurak ML, Sato N, Fukushima N, et al. Serum macrophage inhibitory cytokine 1 as a marker of pancreatic and other periampullary cancers. *Clin Cancer Res* 2004;**10**:2386–2392.
56. Breit SN, Johnen H, Cook AD, Tsai VW, Mohammad MG, Kuffner T, et al. The TGF-beta superfamily cytokine, MIC-1/GDF15: a pleotrophic cytokine with roles in inflammation, cancer and metabolism. *Growth Factors* 2011;**29**:187–195.
57. von Haehling S, Morley JE, Coats AJS, Anker SD. Ethical guidelines for publishing in the Journal of Cachexia, Sarcopenia and Muscle: update 2015. *J Cachexia Sarcopenia Muscle* 2015;**6**:315–316.

6 Original Research Article in preparation: “Central GLP-1 signaling is involved in the mediation of CACS in hepatoma tumor-bearing rats”

The following section contains an original research article that is in preparation and will be submitted to the Journal of Cachexia, Sarcopenia and Muscle for publication.

My contribution to this manuscript includes: conception and design of research, data acquisition, data analysis, data interpretation and writing the manuscript.

Brainstem GLP-1 signalling contributes to cancer anorexia-cachexia syndrome in hepatoma tumour-bearing rats

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Abstract

Background

The cancer anorexia-cachexia syndrome (CACS) is frequent in cancer patients. CACS increases morbidity, mortality and psychological suffering, and compromises treatment success. Currently, no pharmacological treatment is specifically approved and available for the therapy of CACS. Using a rat hepatoma tumour model, we recently demonstrated a role of the *area postrema* (AP) in the mediation of CACS, but the underlying mechanism and possible therapeutic targets still remain to be identified. Glucagon-like peptide-1 (GLP-1) is expressed in neurons of the *nucleus tractus solitarii* (NTS) and is implicated in malaise, nausea and food aversion. GLP-1ergic neurons project from the NTS to the AP where the GLP-1 receptor (GLP-1R) is highly expressed. We hypothesized that NTS/AP dependent GLP-1 signalling might play a role in the mediation of CACS.

Methods

Using hepatoma tumour-bearing (TB) and healthy rats, we first tested whether a pharmacological blockade of brainstem GLP-1 receptors (GLP-1R) via chronic delivery of the GLP-1R antagonist exendin-9 (Ex-9; 100 μg/day) into the fourth ventricle attenuated CACS. Second, we investigated whether a genetic knockdown of GLP-1 expression in the NTS ameliorates CACS. We used a lentiviral short-harpin RNA approach to silence the pre-proglucagon gene (PPG) encoding GLP-1. Finally, using a two diets choice paradigm, we tested if tumour growth in our model is paralleled by the development of food aversion.

Results

In healthy animals, pharmacological and genetic blockade of brainstem GLP-1 signalling did not affect food intake or body weight. In contrast, Ex-9 attenuated anorexia, body weight loss and fat depletion compared to TB controls. Importantly, Ex-9 also ameliorated tumour-induced loss of muscle mass. Similarly, TB animals with a knockdown of GLP-1 expression in the NTS had higher food intake, reduced body weight loss, and higher lean and fat mass compared to NTB controls. TB rats developed tumour-induced food aversion because their flavour preference turned into a flavour rejection during tumour growth.

Conclusions

Our study identifies brainstem GLP-1 as crucial mediator of CACS in hepatoma TB rats. In line with the function of GLP-1 as a mediator of aversion, emesis and nausea, we also demonstrated the development of food aversion during CACS in this model. Based on our current and recent findings, a local release of GLP-1 in the AP appears to contribute to CACS. Since the AP lacks a functional the blood-brain-barrier, the GLP-1R represents a promising target for pharmacological approaches against CACS and possibly other forms of disease-related anorexia/cachexia.

Key terms: cancer, food intake, energy balance, hindbrain, food aversion, muscle.

INTRODUCTION

The cancer anorexia-cachexia syndrome (CACS) causes a decline of the clinical status of cancer patients and often becomes a life-threatening condition [1]. CACS increases mortality, reduces treatment success and produces severe psychological suffering of patients and their families [34, 207]. Its prevalence can reach up to 85% in cancer patients [208]. We recently demonstrated the importance of the *area postrema* (AP) of the brainstem for the mediation of CACS in hepatoma tumour-bearing (TB) rats [209]. In order to identify a possible therapeutic target, our current study aims to explore the role of the neuropeptide glucagon-like peptide-1 (GLP-1) in the brainstem as a mediator of CACS.

GLP-1 is a posttranslational product of the peptide preproglucagon (PPG), which is expressed in enteroendocrine L-cells of the small intestine [115, 210, 211] and in the *nucleus tractus solitarii* (NTS) [127], which is an integrative and relay center for enteroceptive signals controlling food intake [27, 106, 212]. GLP-1-expressing NTS neurons project to the AP [131, 133], which is implicated in the physiological control of food intake, pathological anorexia, nausea, and emesis [87]. The GLP-1 receptor (GLP-1R) is highly expressed in the AP [127, 129, 130].

Systemic as well as central administration of GLP-1 or the GLP-1R agonist exendin-4 reduce food intake and body weight [121-124]. While the possible role of brainstem GLP-1 in the physiological control of energy balance is unclear, GLP-1 signalling is known to induce anorexia, nausea and food aversion in rodents and humans [134-139].

The AP/NTS region is activated in TB rodents [99, 213], by the inflammatory endotoxin LPS**,** by the emetic agent LiCl**,** and by the chemotherapeutic drug cisplatin [143, 214, 215]**.** These and other toxic agents lead to anorexia or malaise, which is reflected by the development of conditioned food aversion (CTA) [106, 216, 217]. LPS and cisplatin-induced anorexia are attenuated in rats receiving fourth ventricular injection of the GLP-1R antagonist exendin-9 (Ex-9) [142, 144]. Central administration of Ex-9 also prevents CTA induced by LiCl [140, 141] and blunts LiCl-induced AP activation [137]. We hypothesize that GLP-1 might also contribute to anorexia and cachectic body weight loss under chronic cancer conditions.

We first examined whether a pharmacological blockade of brainstem GLP-1R by chronic fourth ventricular infusion of Ex-9 blunts CACS. Second, in order to confirm the involvement of local brainstem GLP-1 as a mediator of CACS, we knocked down PPG gene expression in the NTS by a lentiviral short-hairpin RNA (shRNA) approach. Third, we also tested if the development of CACS is paralleled by CTA in our tumour model. Although sickness-related food aversion and anorexia are conceptualized as different phenomena, they often occur together and might act in concert to negatively affect appetite and energy intake.

MATERIALS AND METHODS

Animals and housing conditions

Male Buffalo rats (Charles River Laboratory, USA) were housed at controlled temperature (21±1°C) under a 12-hour artificial light cycle with ad libitum access to standard laboratory rat chow (890 25 W16, Provimi Kliba, AG, Kaiseraugst, Switzerland) except for the CTA studies (see below). All experiments were approved by the Veterinary Office of the Canton Zurich (95/2013).

Cell culture and tumour model

The hepatoma tumour model was described previously [209]. Morris hepatoma 7777 cells (McA-RH7777, Catalog No. CRL-1601, ATCC, USA) were cultured under standard conditions in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. A trypan blue staining was performed to confirm the viability of tumour cells. For tumour induction, 10^7 cells (dissolved in 250 µl PBS) were inoculated subcutaneously between the scapulae under short isoflurane anaesthesia. Control animals were anesthetized and injected with PBS only.

Fourth ventricular cannulation for infusion of Ex-9

Rats (250-280 g) were placed in a stereotactic frame under isoflurane anaesthesia. A guide cannula (9mm 326OPG/Spc, Plastics One, USA) was implanted into the 4th ventricle and anchored by dental cement and stainless screws. The coordinates for cannula placement (bregma -11.6 mm, lateral 0.0 mm, dorsoventral -7.2 mm) were chosen according to the rat brain atlas of Paxinos and Watson [218]. The rats were treated with antibiotics (7.5 mg/kg, Baytril®), prednisolone (25 µg/kg, Prednisolut®) and analgesic (1 mg/kg, Norocarp®). After recovery, the correct placement of the cannula was verified by measuring the hyperglycaemic response to fourth ventricular infusion of 5-thio-D-glucose (5TG, 210 µg 5TG dissolved in 3 µl of saline). Only animals with a correct cannula placement display elevated plasma glucose levels [219]. Animals that did not show an increase of at least 100% in their plasma glucose concentration 30 min after 5TG injection were excluded from the experiment.

Measurement of energy expenditure

The experiments were conducted in an open-circuit indirect calorimetric system (TSE Phenomaster, Bad Homburg, Germany) equipped with internal food hoppers for continuous recording of food consumption. The animals were single-housed in metabolic cages and adapted to the housing conditions for 7 days before the start of the experiment. Body weight was measured daily before dark onset. Food intake and respiratory gas exchange (O_2 and CO_2) were recorded automatically at 17-min intervals throughout the entire experiment.

First, we investigated the effect of chronic central Ex-9 treatment on food intake, body weight, metabolic rate and respiratory exchange ratio (RER) in non-tumour-bearing (NTB) rats. After baseline measurements, primed osmotic minipumps (model 2ML2, ALZET, DURECT corporation, USA) were implanted subcutaneously into the left flank between the chest and the hind limbs and connected to the forth ventricular cannula via a subcutaneous vinyl catheter (C312VT, Plastics One, USA). Minipumps were filled with Ex-9 or saline for controls. The minipumps released a constant amount of Ex-9 (100 µg/day) directly into the 4th ventricle. Similar experiments were conducted in

hepatoma tumour-bearing rats. After baseline measurements, tumour cells were inoculated in two groups of TB animals receiving Ex-9 or saline treatment as described above. Minipumps were implanted on day 9 after tumour inoculation, i.e. at the typical onset of the anorectic response [209, 220]. The minimump infusion lasted for 11 days until the animals were euthanized according to the ethical criteria for the termination of the experiments. Animals were euthanized for tissue collection and carcass analysis with a lethal intraperitoneal dose of pentobarbital (100 mg/kg) injected shortly before dark onset (see below).

Lentiviral vectors

High-titer (>10^9 IU/ml) lentiviral vectors dissolved in PBS, produced by Sigma-Aldrich, were gently provided by Dr. Shin Lee, ETHZ, Zurich, Switzerland. These viral vectors expressed either a non-target sequence (control) shRNA or a shRNA directed against the rat PPG mRNA under the control of the human U6 promoter. In addition, the vectors contained an independent enhanced green fluorescent protein (turboGFP) cassette for the detection of transduced neurons. The PPG shRNA oligonucleotide sequence was *tgccaaacgtcatgatgaatt*.

Lentiviral shRNA knockdown of PPG gene expression in the NTS

Rats (260-320 g) were anesthetized with an ip injection of 50mg/kg ketamine and 6mg/kg xylazine and placed in a stereotactic frame with the head in a ventroflexed position. A 2 cm midline incision was made above the occipito-atlantal joint, the muscles were retracted and the joint capsule was opened. For the injections, a 10µl glass-syringe (Nanofil, World Precision Instrument, USA) equipped with a 35G bevelled needle (World Precision Instrument, USA) was attached to a stereotactic arm. Using a microinfusion pump (World Precision Instrument, USA), 2.0 µl of virus solution were infused bilaterally under visual control into the NTS over 20 min (400 µm lateral and 100 µm caudal to the obex, and 600 µm below the surface of the brain). These coordinates were chosen to target the area of the NTS with the highest density of PPG

neurons [221, 222]. To prevent reflux of virus solution, the needle was left in position for 10 min after the infusion. The needle was then removed and the skin sutured. Post-surgical treatments were the same as described above.

Three weeks after the lentivirus injection, rats were single-housed and adapted to the housing conditions for 14 days before tumour cell injection. PPG knockdown (PPG-KD) and control animals were kept in cages equipped with external food hoppers for manual measurement of food intake (Research Diets, NJ, USA). Daily food intake and body weight were measured daily shortly before dark-onset. At the end of the experiment, animals were euthanized for tissue collection and carcass analysis with a lethal dose of pentobarbital (see above).

Tissue collection, qPCR, and confirmation of PPG knockdown

After sacrifice, the caudal medulla was dissected and snap-frozen in liquid nitrogen. Samples were stored at -80°C until processing. Micro-punch samples (2 mm diameter) containing the AP/NTS region were obtained under a dissecting microscope on 3 consecutive 250 µm-thick coronal sections previously cut on a cryostat (Leica microsystem, Germany) and pooled together. Sections were collected starting 250 µm caudal to the obex. RNA was extracted and purified according to the manufacturer's instructions (ReliaPrep, Promega, USA). The concentration and integrity of RNA were measured using a nanodrop system (NanoDrop 1000 Spectrophotometer, Thermo Scientific). cDNA was generated from the extracted RNA using the high capacity cDNA reverse transcription kit (Applied Biosystems, USA). Quantitative polymerase chain reaction was performed using the 7500 Fast system (Applied Biosystem/Life technologies) with pre-designed Taqman probes (Thermoscientific). Rat S18 (Ref. nr. Rn01428913) was used as housekeeping reference gene and the following probes were used to amplify PPG (Ref. nr. Rn00562293), GLP-1R (Ref. nr. Rn00562406), IL-1β (Ref. nr. Rn00580432), TNF-α (Ref. nr. Rn01525860), and IFN-γ (Ref. nr. Rn00594078) transcripts. The relative expression of PPG, GLP-1R, TNF-α, IL-1β, and IFN-γ mRNA was calculated using the comparative delta-delta Ct method [223]. Each sample was run in duplicates.

Measurements of brainstem PPG and GLP-1R expressions, and circulating GLP-1 levels

PPG and GLP-1R mRNA expression levels were analysed in non-operated TB, NTB, and NTB/pair-fed (PF) rats (220-250 g). Moreover, to evaluate a possible contribution of peripheral GLP-1 signalling in the mediation of CACS, circulating GLP-1 levels were also measured. For this experiment rats were single-housed as described above. Daily food intake and body weight were measured shortly before dark-onset. Starting from nine days after tumour induction of the TB group, PF animals received the same amount of food that was consumed the previous day by the TB rats. At the end of the experiment (i.e. 19 days after tumour-induction) all animals were euthanized and brains were immediately dissected and processed for gene expression analysis as described above. Blood was collected from the right ventricle of the heart in EDTA-coated tubes (Sarstedt, Germany) containing a DPP-IV inhibitor (1:100, Millipore, USA) and centrifuged at 7'000 x g (4°C, 7 min) to obtain plasma which was stored in aliquots at -80°C for subsequent GLP-1 measurements. The levels of GLP-1 were measured in triplicates using a commercial kit (Meso Scale Discovery, USA) according to the manufacturer instructions.

Computed tomography (CT) scanning and muscle weight measurements

Total carcass lean and fat volumes were measured by quantitative microcomputed tomography (La Theta LCT-100A scanner, Hitachi-Aloka Medical, Japan) as previously described [220]. Sequential 2-mm slice images with a pixel size of 250 × 250 μm were used for calculations using LaTheta software (version 2.10). Head, lungs and tail were excluded from the analysis of tissue volumes. After the CT scanning, the left gastrocnemius, tibialis and soleus muscles were dissected at the level of their upper to lower tendons and weighed.

Food aversion study

A two diets choice paradigm was used. The diets had identical nutrient composition and caloric content, but different flavours (AIN-76A-modified diets with either 0.4% chocolate or 0.4% vanilla flavour, respectively, Research Diets, US). Buffalo rats (300-340 g) were single-housed in BIODAQ cages equipped with 2 external food hoppers for the parallel measurement of diet consumption. First, taste preference for the two diets was determined before tumour induction by measuring the relative daily food intake over a 4-days period. For the following 7 days, the animals received standard chow only (AIN-76A, Research Diet, US). Two days after the food preference test, in half of the animals, tumour growth was induced, while in control animals PBS was injected. Before the expected onset of the anorectic response (i.e. 5 days after tumour induction [the anorectic response typically starts around day 9; [209, 220]]), all animals received their individually preferred flavoured diet (i.e. mean relative consumption during the food preference test >50%) ad libitum for 8 days. During the anorectic phase (14 days after tumour induction), the standard diet was presented again for 2 days before a final preference test was conducted by presenting both flavoured diets over 5 consecutive days.

Data evaluation and statistical analysis

All data were expressed as mean ± SEM. In the Ex-9 studies, body weight changes were calculated by subtracting the weight of the animal at the day of minipump implantation from the final body weight. These values were corrected for the weight of the minipumps. In the experiments involving PPG-KD animals, body weight change was calculated by subtracting the body weight before the onset of the anorectic response (day 8 after tumour induction) from the body weight at the end of the experiment. Metabolic rate and RER were calculated from O_2 consumption and CO_2 production as described previously [220]. Metabolic rate data were normalized for body weight. The calculation was based on the following equation: total EE (kcal/kg/h) = (3.9 x VO_2 + 1.1 x VCO_2) / 1000. For the experiment involving Ex-9 treatment in healthy rats, the average values obtained from 2 consecutive days prior to minipump implantation were

used as baseline and compared to the average values of treatment days 3 and 4. In the experiment involving TB animals treated with Ex-9, the values obtained between days 2-3 after tumour inoculation were used as baseline (i.e., prior to the onset of anorexia) and compared to the average values on days 12-13 after tumour induction (i.e. treatment days 3-4). In the food aversion study, diet preference was calculated for each animal by dividing the consumption of the preferred diet by total daily food intake.

Unpaired Student's *t*-test was used for comparison between two groups. Statistical comparisons between multiple groups were performed using one-way ANOVA followed by Tukey's post-hoc test. For all statistical tests, a *p*-value less than 0.05 was considered significant. Data were analysed using Prism GraphPad 5.0.

RESULTS

Chronic fourth ventricular infusion of Ex-9 did not affect energy balance in non-tumour-bearing rats

Chronic 12-day infusion of Ex-9 into the fourth ventricle did not affect daily food intake (Fig. 1A-B), body weight and body weight gain (Fig. 1 C-D) compared to control animals. Similarly, no change in body composition occurred between Ex-9 treated rats compared to controls, and Ex-9 treated and control animals did not differ in body composition (data not shown). Moreover, metabolic rate and RER were similar in both groups and no significant changes occurred during treatment (Fig. 1E-F).

Chronic fourth ventricular infusion of Ex-9 attenuated CACS in hepatoma tumour-bearing rats

No significant difference in food intake between the experimental groups occurred prior to minipump implantation. Tumours became palpable around day 9 after tumour induction, which typically coincides with the approximate onset of the anorectic response in this tumour model. Vehicle-treated TB (Vehicle/TB) animals showed a clear anorectic response. In Ex-9-treated TB (Ex-9/TB) rats, anorexia was markedly attenuated compared to controls (Figure 2A). On average, daily food intake during the treatment period (i.e. from day 10 to day 20 after tumour induction) was 16.3±0.4 g vs. 13.9±0.6 g (Ex-9/TB vs. Vehicle/TB; $p < 0.01$), which corresponds to 17% higher food intake of Ex-9/TB rats compared to controls (Figure 2B). Tumour-induced body weight loss was attenuated in rats receiving Ex-9. While the difference in absolute body weight did not reach statistical significance, the change in body weight was significantly different between treatment groups (Figure 2C). This appeared to be mainly due to a preservation of fat mass because Ex-9/TB rats had higher fat mass but similar total lean mass compared to Vehicle/TB animals (Figure 2D). However, Ex-9/TB rats had higher gastrocnemius and total hind limb muscle weights (i.e. sum of gastrocnemius, tibialis and soleus muscle mass) than TB controls (Fig. 2E). Relative to baseline conditions, i.e. before the onset of anorexia on days 2-3 after tumour induction, control rats had

significantly lower RER values on days 12-13 reflecting an increased lipid metabolism. While the tumour-dependent reduction in RER was not observed in the Ex-9/TB group, the RER of these animals did not significantly differ from the vehicle treated group (Fig. 3A). The metabolic rate was similar between Ex-9/TB and Vehicle/TB animals before and during the treatment period (Fig. 3B). Importantly, tumour weight did not differ significantly between Ex-9/TB and Vehicle/TB rats at the end of the experiment (17.5±1.1 vs. 14.2±1.2 g).

Knockdown of PPG expression in the NTS attenuated CACS

Fig. 4A shows a representative histological photomicrograph of the right caudal NTS displaying GFP fluorescence 7 weeks after the lentiviral vector injection. The indicated target region (Fig. 4B) corresponds to the caudal NTS area where PPG neurons are located [222]. Seven weeks after the lentivirus injection, PPG-KD rats expressed significantly (-47%) less PPG mRNA in the AP/NTS region than controls (Fig. 4C). No change in GLP-1R expression occurred between the experimental groups (Fig. 4D). Furthermore, mRNA levels of TNF-α, IL-1β, and IFN-γ in the AP/NTS did not differ between PPG-KD and controls. The relative expression values were comparable to non-operated TB and NTB rats indicating the absence of local virus-induced inflammation (data not shown).

Both experimental groups showed similar food intake in the week prior to tumour cell inoculation (PPG-KD: 23.0±1.0 g/day vs. controls 23.5±1.0 g/day) and prior to tumour anorexia (Fig 5A). Food intake of control animals decreased 10 days after tumour induction leading to a 30% reduction on the last day of the experiment compared to baseline (i.e. days 1-2 after tumour induction, Fig. 5A). On average, mean daily food intake was 26% higher in PPG-KD than controls from day 8 to the end of the experiment (Fig. 5C). Anorexia in control animals was associated with marked loss of body weight. This effect was significantly attenuated in PPG-KD animals beginning at day 13 (Fig. 5B-D). The body weight change between day 8 after tumour induction and the end of the experiment was -6.4±2.7 g vs. -23.9±4.7 g (PPG-KD vs. controls). The differences in body weight were due to both higher lean and fat volumes (Fig. 5E). Despite the higher lean mass of the PPG-KD group, differences in hind limb muscle mass did not reach statistical

significance (not shown). Tumour weight did not differ significantly between the two groups (PPG-KD: 11.9±1.1 vs. controls: 12.1±0.9 g).

Tumour growth decreased PPG expression without affecting circulating GLP-1 and GLP-1R expression in the AP/NTS.

Circulating active GLP-1 levels were not significantly different between NTB, PF and TB rats (Fig. 6A). Tumour-bearing animals had lower PPG expression compared to NTB rats. However, pair-feeding also reduced PPG expression to the level of the TB animals (Fig. 6B). No significant difference in GLP-1R expression levels occurred between the experimental groups.

Hepatoma tumour-bearing rats developed a conditioned taste aversion during cancer anorexia

TB rats showed lower food intake than NTB animals from day 8 after tumour cell inoculation. Daily food intake stabilized at a lower level with an average reduction of 30% in comparison to NTB animals (Fig. 7B). In both experimental groups, alteration of the diet's flavour properties did not affected total food consumption.

In the first diet choice test (i.e. prior to tumour induction), animals exhibited a bimodal pattern of preference (i.e., 8 animals preferred the vanilla flavour diet and 7 the chocolate flavour diet (87% and 80% of total intake over the 4 testing days; respectively). The relative flavour preference values remained constant during 4 consecutive testing days (Fig. 7C). Vanilla and chocolate preferring rats were allocated in similar numbers to both experimental groups (TB and NTB). NTB animals showed constant food intake and a constant body weight gain during the entire experimental period. In TB rats, food intake decreased 8 days after tumour induction (Fig. 7B), leading to significant body weight loss compared to NTB (data not shown). During the second preference test, healthy controls had a similar flavour preference as in the first trial before tumour induction. TB rats clearly avoided the flavour that was preferred before tumour growth and presented during the conditioning phase (Fig. 7D).

DISCUSSION

The present study extends our previous work that established a role of the AP in a rat hepatoma tumour model of CACS [209]. We identified GLP-1 as a neurochemical mediator of brainstem-dependent CACS providing first evidence that the central GLP-1 system mediates cancer anorexia and subsequent body weight loss. A pharmacological and genetic blockade of GLP-1R signalling in the brainstem attenuated anorexia and body weight loss induced by tumour growth. Notably, the blockade of brainstem GLP-1R by Ex-9 also ameliorated muscle wasting. In line with the function of GLP-1 as a mediator of aversion, emesis and nausea, we also demonstrated the development of food aversion during CACS in hepatoma TB rats.

In several studies, endogenous GLP-1 signalling was inhibited in the brainstem to study its involvement in energy balance. At least in freely feeding rats a blockade of brainstem GLP-1R does not affect energy intake or body weight development [144, 224, 225]. Our findings are consistent with these studies because neither GLP-1R blockade nor PPG-KD affected food intake or body weight in NTB rats. Hence, brainstem GLP-1 does not seem to be required for the maintenance of energy balance under standard experimental conditions in healthy animals. Consequently, the anti-CACS effects described in our experiments are due to a specific blockade of a pathological activation of the GLP-1 system, but not secondary to a change in basal energy balance.

Although peripherally administered GLP-1 reduces food intake and body weight [125, 226-228], our findings do not suggest a role of peripheral GLP-1 in the mediation of CACS. There was only a tendency of increased circulating GLP-1 in TB rats but the levels were not significantly different from NTB or pair-fed animals. Intestinal GLP-1 might also act locally on GLP-1R on terminals of intestinal vagal afferents [226, 229]. However, according to our recent studies, vagal afferents are not required for CACS in this tumour model [209]. Our current study rather supports a role of brainstem GLP-1 and local GLP-1R in the mediation of CACS.

The AP and the lateral parabrachial nucleus (LPBN) are important first order projection sites of NTS GLP-1 neurons in the brainstem [131]. Fourth ventricular Ex-9 is unlikely to block GLP-1R in the LPBN or in the forebrain because of the rostral-to-caudal flow of the cerebrospinal fluid [144, 230]. Therefore, CACS appears to be driven by GLP-1-ergic

projections from the NTS to the AP. This is also consistent with the similarity of our current findings and the outcome of our recent AP lesion studies [209].

The results of our pharmacological and genetic blockade of brainstem GLP-1 signalling were similar. In both approaches, we observed either a positive treatment effect on lean mass or muscle mass in addition to a preservation of fat mass that occurred under both experimental conditions. It might appear surprising that the positive effect of Ex-9 treatment on hind limb muscle mass was not paralleled by increased whole body lean mass. Vice versa, PPG-KD rats had higher total lean mass but no increased hind limb muscle mass. Apparently, these parameters do not necessarily correspond, at least not in the time frame of our experiments. It should be mentioned, however, that PPG-KD animals were sacrificed 4 days earlier than those in the Ex-9 experiment. Hence, muscle atrophy might not have been advanced at that stage, which could account for the lack of a statistically significant treatment effect on muscle mass. Additionally, CACS is associated with sarcopenic obesity during which lipids are stored in inappropriate depots (e.g. in muscle). Whether abnormal intramuscular fat deposition (i.e. myosteatosis) occurred in our model was not evaluated. However, one can speculate that a reduction of tumour-induced intramuscular fat deposition following Ex-9 treatment might mask the protective effects of Ex-9 on muscle fibers. A functional test (e.g. grip strength) in these animals could be used to determine whether Ex-9 treatment results in changes in muscle function. Nonetheless, our findings are in principle consistent with our recent demonstration that brainstem-dependent mechanisms contribute to cancer anorexia, muscle wasting and loss of fat and lean body mass [209].

We have not yet explored the possible anti-CACS effect of peripheral Ex-9 administration in TB rats. Due to the absence of a functional blood-brain barrier AP neurons are accessible by blood-borne substances [83] that typically do not cross the blood-brain barrier. This favours pharmacological approaches targeting GLP-1R in the AP. While GLP-1R agonists are therapeutically approved for the treatment of obesity and diabetes [231], we are not aware of a therapeutic GLP-1R antagonist.

Under our current experimental conditions, the anti-CACS effect of GLP-1 blockade appears to be mainly based on an improvement of energy intake, leading to a preservation of fat mass and ultimately also lean mass. A blockade of brainstem GLP-1 signalling did not affect the metabolic rate. The cachectic effect in hepatoma TB rats is partly independent of reduced energy intake because TB rats loose more body weight

and muscle mass than pair-fed NTB animals [209]. Therefore, impaired energy expenditure appears to contribute to CACS in this tumour model. While we did not explore the effects of peripheral Ex-9 administration on CACS, additional beneficial effects might occur in the periphery also with respect to energy balance. At least in diet-induced obese mice, peripheral treatment with a GLP-1R agonist increased basal energy expenditure presumably via increased expression of uncoupling proteins in different tissues including muscle [232]. Moreover the GLP-1R agonist exendin-4 increased oxygen consumption and thermogenic gene expression in muscle cells, which can be blocked by Ex-9 [233]. Antagonism of these effects might be beneficial to preserve energy and muscle mass in parallel to the centrally mediated attenuation of anorexia. Whether peripheral treatment with a GLP-1R antagonist such as Ex-9 counteracts hypermetabolism via such peripheral mechanisms under cancer conditions remains to be elucidated.

GLP-1R expression levels in the AP/NTS were unaffected by tumour growth or pair-feeding. Quite surprisingly, tumour growth decreased PPG expression levels in the NTS. However, no difference was found between PF and TB animals, indicating that the decrease in PPG expression during tumour growth is likely due to change in eating rather than specific tumour -dependent effects.
Despite the lower NTS GLP-1 expression levels following tumour growth, GLP-1 blockade was effective in attenuating CACS. Changes in PPG mRNA expression may not necessarily correlate with GLP-1 abundance or GLP-1 synaptic release. Further studies are required to investigate this interesting aspect.

The brainstem GLP-1 system mediates acute behavioural sickness responses induced by LPS, LiCl und cisplatin [141, 142, 144]. Food aversion following malaise is a commonly known phenomenon, which not only occurs under these experimental conditions but also in cancer patients [106, 234]. The development of CTA can be a consequence of the cancer itself but also secondary to anti-cancer treatment such as chemotherapy and irradiation [234-239]. The mechanisms contributing to taste abnormalities are multifactorial and involve direct alterations in taste sensing and perception or conditioned avoidance food properties unrelated to impaired taste responses [106, 238,

240-242]. Irrespective of their cause, taste abnormalities and aversions may contribute to and worsen the loss of appetite and anorexia in cancer patients.
Our findings may suggest a GLP-1 dependent mediation of CTA, which could be assessed by combining a CTA study with GLP-1 blockade. Whether tumour-dependent aversion contributes to anorexia is difficult to address under our experimental conditions. The development of anorexia during our CTA experiment was not affected by changing the diet or by giving the choice between two flavours during the final preference test. Hence, at least in our studies, alteration of the diet's flavour properties did not counteract anorexia. It remains to be determined whether the attenuation of anorexia and body weight loss by a blockade of brainstem GLP-1 signalling is partly secondary to a suppression of aversive mechanisms.

Although we identified GLP-1 as neuronal mediator of CACS in the AP/NTS region, it remains unknown how NTS GLP-1 neurons are activated under tumour conditions. Recent studies suggest a role of the macrophage inhibitory cytokine-1 (MIC-1) in CACS, which acts via the AP/NTS region to reduce food intake and body weight [99, 101]. Our model is characterized by increased levels of MIC-1 in the absence of other inflammatory circulating cytokines [209, 213]. MIC-1 primarily activates non-catecholaminergic neurons in the NTS [101]. GLP-1-expressing neurons are not catecholaminergic [222], but it is currently unknown whether MIC-1 activates GLP-1 expressing NTS neurons. The dendrites of NTS PPG neurons extend into the AP and to the border of the fourth ventricle. Therefore, NTS neurons could directly respond to factors in the blood and cerebrospinal fluid [131]. This may lead to GLP-1 release in the AP and a subsequent activation of GLP-1R expressing neurons (Fig. 8).

In summary, we found GLP-1/GLP-1R signalling in the brainstem to be required for the central mediation of CACS in hepatoma TB rats. A blockade of brainstem GLP-1 not only attenuated anorexia, but also body weight loss and muscle wasting. Future studies should address the question whether brainstem GLP-1 might mediate anorexia and cachexia in other cancer or chronic disease models. Moreover, it remains to be established which stimuli activate the brainstem GLP-1 system under these sickness conditions. Based on our findings GLP-1R antagonists may be therapeutically useful for the treatment of CACS and possibly other form of sickness-anorexia.

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Conflict of interest:

All authors declare that they have no conflict of interest.

Figure legends:

Fig. 1 Chronic central Ex-9 treatment did not affect energy balance in healthy rats

Chronic fourth ventricular infusion of Ex-9 infusion (100 μg/day) did not affect food intake (A-B), body weight or body weight gain (C-D) in non-tumour-bearing rats. (E-F) Ex-9 treatment did not alter respiratory exchange ratio and metabolic rate. Baseline data were collected for two consecutive days prior to minipump implantation. Data analysed with Student's *t*-test (A-D) or with one-way ANOVA followed by Tukey's post-hoc test (E-F). Different letters indicate significant differences ($p<0.05$).

Fig. 2 Chronic central Ex-9 treatment attenuated anorexia, body weight loss, fat and muscle wasting induced by tumour growth

A-B) Ex-9 (100 μg/day) administered chronically into the fourth ventricle significantly attenuated the anorectic response induced by tumour growth. C) Ex-9 treated rats showed attenuated tumour-dependent body weight loss following treatment. Ex-9 treated animals had higher fat mass than controls (D) and higher gastrocnemius, and total muscle mass (sum of gastrocnemius, tibialis and soleus muscle mass) compared to tumour-bearing controls (E). Data analysed using the Student's *t*-test, * $p < 0.05$, ** $p < 0.01$.

Fig. 3 Chronic central Ex-9 treatment did not affect respiratory exchange ratio and metabolic rate in hepatoma tumour-bearing rats

A) Following tumour induction, control rats had significantly lower RER values relative to baseline conditions, reflecting a shift towards lipid metabolism. Ex-9 treatment (100μg/day) did not prevent this tumour-induced reduction. B) Metabolic rate did not significantly differ between groups. Data analysed with one-way ANOVA followed by Tukey's post-hoc test. Means with different letter are significantly different from each other ($p<0.05$).

Fig. 4 Lentiviral PPG-specific shRNA injection into the NTS significantly decreased PPG mRNA expression

A) Representative image of GFP fluorescence in the NTS seven weeks after lentivirus infusion, indicating successful transfection. Scale bar 50 μm. B) Location of NTS target area for lentiviral PPG knockdown. Bregma level -14.64. Gr, gracile nucleus; SolC, commissural nucleus of the solitary tract; Sol, nucleus of the solitary tract; 10, dorsal motor nucleus of the vagus; CC, central canal. Modified image from The Rat Brain in Stereotaxic Coordinates, Paxinos, G. and Watson, C., Copyright Paxinos, G. and Watson, C. (1998), with permission from Elsevier. C-D) Quantification of PPG and GLP-1R mRNA levels in the AP/NTS of rats injected with lentivirus encoding for a PPG-specific shRNA or control lentivirus.

Fig. 5 Knockdown of PPG mRNA expression in the NTS attenuated anorexia, and loss of body weight by preservation of lean and fat mass

A-B) PPG knockdown in the NTS strongly attenuated anorexia and body weight loss induced by tumour growth without affecting baseline food intake before tumour induction. C-D) From day 8 onwards, PPG KD animals showed significantly increased food intake and lost significantly less body weight compared to controls. E) The higher body weight of PPG KD animals was due to by higher lean and fat volumes compared to tumour-bearing controls. Data analysed using the Student's *t*-test, $* p < 0.05$, $** p < 0.01$, $*** p < 0.001$.

Fig. 6 Tumour growth did not affect circulating GLP-1, and PPG and GLP-1R expressions levels in the AP/NTS

A) Circulating GLP-1 did not significantly differ between groups. B) Tumour-bearing rats (TB) and pair-fed animals (PF) had lower PPG expression levels compared to non-tumour-bearing controls (NTB). C) GLP-1R expression was similar among all groups. Data analysed with one-way ANOVA followed by Tukey's post-hoc test. Means with different letter are significantly different from each other ($p<0.05$).

Fig. 7 Tumour growth induced food aversion

A) Experimental paradigm used to evaluate the role of food aversion following tumour growth. B) Tumour-bearing (TB) rats developed anorexia 8 days after tumour induction. In both tumour-bearing and non-tumour-bearing (NTB) animals, alteration of the diet's flavour properties did not affected total food consumption. C) Prior to tumour induction the two groups displayed similar preference values that remained constant during consecutive testing days. C) After tumour induction, tumour-bearing animals displayed a marked reduction in preference for the diet that was presented during tumour growth compared to non-tumour-bearing rats. This food avoidance persisted for the entire experimental period. Data analysed using the Student's *t*-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 8 Model of GLP-1-dependent mediation of CACS during tumour growth

Humoral mediators such as MIC-1 may directly or indirectly activate GLP-1 expressing neurons located in the NTS leading to GLP-1 release in the AP and activation of GLP-1R expressing neurons. Neuronal efferents of the AP activate neurons in downstream projection sites involved in the control of energy balance.

REFERENCES:

1. Laviano A, Meguid MM, Rossi-Fanelli F. Cancer anorexia: clinical implications, pathogenesis, and therapeutic strategies. Lancet Oncol. 2003;4(11):686-94.
2. Gordon JN, Green SR, Goggin PM. Cancer cachexia. Qjm. 2005;98(11):779-88.
3. Tisdale MJ. Cachexia in cancer patients. Nat Rev Cancer. 2002;2(11):862-71.
4. Tisdale MJ. Biology of cachexia. J Natl Cancer Inst. 1997;89(23):1763-73.
5. Borner T, Arnold M, Ruud J, Breit SN, Langhans W, Lutz TA, Blomqvist A, Riediger T. Anorexia-cachexia syndrome in hepatoma tumour-bearing rats requires the area postrema but not vagal afferents and is paralleled by increased MIC-1/GDF15. Journal of cachexia, sarcopenia and muscle. 2016.
6. Holst JJ. The physiology of glucagon-like peptide 1. Physiol Rev. 2007;87(4):1409-39.
7. Eissele R, Goke R, Willemer S, Harthus HP, Vermeer H, Arnold R, Goke B. Glucagon-like peptide-1 cells in the gastrointestinal tract and pancreas of rat, pig and man. Eur J Clin Invest. 1992;22(4):283-91.
8. Hansen CF, Vrang N, Sangild PT, Jelsing J. Novel insight into the distribution of L-cells in the rat intestinal tract. Am J Transl Res. 2013;5(3):347-58.
9. Merchenthaler I, Lane M, Shughrue P. Distribution of pre-pro-glucagon and glucagon-like peptide-1 receptor messenger RNAs in the rat central nervous system. J Comp Neurol. 1999;403(2):261-80.
10. Grill HJ, Hayes MR. The nucleus tractus solitarius: a portal for visceral afferent signal processing, energy status assessment and integration of their combined effects on food intake. Int J Obes (Lond). 2009;33 Suppl 1:S11-5.
11. Bernstein IL. Taste aversion learning: a contemporary perspective. Nutrition. 1999;15(3):229-34.
12. Grill HJ, Hayes MR. Hindbrain Neurons as an Essential Hub in the Neuroanatomically Distributed Control of Energy Balance. Cell Metab. 2012;16(3):296-309.
13. Llewellyn-Smith IJ, Reimann F, Gribble FM, Trapp S. Preproglucagon neurons project widely to autonomic control areas in the mouse brain. Neuroscience. 2011;180:111-21.
14. Jin SL, Han VK, Simmons JG, Towle AC, Lauder JM, Lund PK. Distribution of glucagonlike peptide I (GLP-I), glucagon, and glicentin in the rat brain: an immunocytochemical study. J Comp Neurol. 1988;271(4):519-32.
15. Price CJ, Hoyda TD, Ferguson AV. The area postrema: a brain monitor and integrator of systemic autonomic state. Neuroscientist. 2008;14(2):182-94.
16. Dunphy JL, Taylor RG, Fuller PJ. Tissue distribution of rat glucagon receptor and GLP-1 receptor gene expression. Mol Cell Endocrinol. 1998;141(1-2):179-86.
17. Shimizu I, Hirota M, Ohboshi C, Shima K. Identification and localization of glucagon-like peptide-1 and its receptor in rat brain. Endocrinology. 1987;121(3):1076-82.
18. Hayes MR, De Jonghe BC, Kanoski SE. Role of the glucagon-like-peptide-1 receptor in the control of energy balance. Physiol Behav. 2010;100(5):503-10.
19. Hayes MR. Neuronal and intracellular signaling pathways mediating GLP-1 energy balance and glycemic effects. Physiol Behav. 2012;106(3):413-6.
20. Turton MD, O'Shea D, Gunn I, Beak SA, Edwards CM, Meeran K, Choi SJ, Taylor GM, Heath MM, Lambert PD, Wilding JP, Smith DM, Ghatei MA, Herbert J, Bloom SR. A role for glucagon-like peptide-1 in the central regulation of feeding. Nature. 1996;379(6560):69-72.
21. Williams KW, Elmquist JK. From neuroanatomy to behavior: central integration of peripheral signals regulating feeding behavior. Nat Neurosci. 2012;15(10):1350-5.
22. Garber AJ. Long-acting glucagon-like peptide 1 receptor agonists: a review of their efficacy and tolerability. Diabetes Care. 2011;34 Suppl 2:S279-84.
23. Marre M, Shaw J, Brandle M, Bebakar WM, Kamaruddin NA, Strand J, Zdravkovic M, Le Thi TD, Colagiuri S. Liraglutide, a once-daily human GLP-1 analogue, added to a sulphonylurea over 26 weeks produces greater improvements in glycaemic and weight control compared with adding rosiglitazone or placebo in subjects with Type 2 diabetes (LEAD-1 SU). Diabet Med. 2009;26(3):268-78.
24. Calara F, Taylor K, Han J, Zabala E, Carr EM, Wintle M, Fineman M. A randomized, open-label, crossover study examining the effect of injection site on bioavailability of exenatide (synthetic exendin-4). Clin Ther. 2005;27(2):210-5.
25. Thiele TE, Seeley RJ, D'Alessio D, Eng J, Bernstein IL, Woods SC, van Dijk G. Central infusion of glucagon-like peptide-1-(7-36) amide (GLP-1) receptor antagonist attenuates lithium chloride-induced c-Fos induction in rat brainstem. Brain Res. 1998;801(1-2):164-70.

26. Van Dijk G, Thiele TE, Donahey JC, Campfield LA, Smith FJ, Burn P, Bernstein IL, Woods SC, Seeley RJ. Central infusions of leptin and GLP-1-(7-36) amide differentially stimulate c-FLI in the rat brain. Am J Physiol. 1996;271(4 Pt 2):R1096-100.
27. Kinzig KP, D'Alessio DA, Seeley RJ. The diverse roles of specific GLP-1 receptors in the control of food intake and the response to visceral illness. J Neurosci. 2002;22(23):10470-6.
28. Ruud J, Blomqvist A. Identification of rat brainstem neuronal structures activated during cancer-induced anorexia. J Comp Neurol. 2007;504(3):275-86.
29. Johnen H, Lin S, Kuffner T, Brown DA, Tsai VW, Bauskin AR, Wu L, Pankhurst G, Jiang L, Junankar S, Hunter M, Fairlie WD, Lee NJ, Enriquez RF, Baldock PA, Corey E, Apple FS, Murakami MM, Lin EJ, Wang C, During MJ, Sainsbury A, Herzog H, Breit SN. Tumor-induced anorexia and weight loss are mediated by the TGF-beta superfamily cytokine MIC-1. Nat Med. 2007;13(11):1333-40.
30. Rinaman L. Interoceptive stress activates glucagon-like peptide-1 neurons that project to the hypothalamus. Am J Physiol. 1999;277(2 Pt 2):R582-90.
31. Endo T, Minami M, Nakayasu M, Hirafuji M, Hamaue N, Omae N, Kang YN, Iwanaga T. Effects of granisetron and vagotomy on c-fos mRNA expression in the rat medulla oblongata as assessed by in situ hybridization. Biomed Res-Tokyo. 2004;25(5):229-35.
32. Horn CC, Ciucci M, Chaudhury A. Brain Fos expression during 48 h after cisplatin treatment: neural pathways for acute and delayed visceral sickness. Autonomic neuroscience : basic & clinical. 2007;132(1-2):44-51.
33. Yamamoto T, Nagai T, Shimura T, Yasoshima Y. Roles of chemical mediators in the taste system. Japanese journal of pharmacology. 1998;76(4):325-48.
34. Mele PC, McDonough JR, McLean DB, O'Halloran KP. Cisplatin-induced conditioned taste aversion: attenuation by dexamethasone but not zacopride or GR38032F. European journal of pharmacology. 1992;218(2-3):229-36.
35. Grill HJ, Carmody JS, Amanda Sadacca L, Williams DL, Kaplan JM. Attenuation of lipopolysaccharide anorexia by antagonism of caudal brain stem but not forebrain GLP-1-R. Am J Physiol Regul Integr Comp Physiol. 2004;287(5):R1190-3.
36. De Jonghe BC, Holland RA, Olivos DR, Rupprecht LE, Kanoski SE, Hayes MR. Hindbrain GLP-1 receptor mediation of cisplatin-induced anorexia and nausea. Physiol Behav. 2016;153:109-14.
37. Seeley RJ, Blake K, Rushing PA, Benoit S, Eng J, Woods SC, D'Alessio D. The role of CNS glucagon-like peptide-1 (7-36) amide receptors in mediating the visceral illness effects of lithium chloride. J Neurosci. 2000;20(4):1616-21.
38. Rinaman L. A functional role for central glucagon-like peptide-1 receptors in lithium chloride-induced anorexia. Am J Physiol. 1999;277(5 Pt 2):R1537-40.
39. Paxinos G, Watson C. The Rat Brain in Stereotactic Coordinates. 6th Edition ed. Elsevier; 2006.
40. Ritter RC, Slusser PG, Stone S. Glucoreceptors controlling feeding and blood glucose: location in the hindbrain. Science. 1981;213(4506):451-2.
41. Borner T, Loi L, Pietra C, Giuliano C, Lutz TA, Riediger T. The ghrelin receptor agonist HM01 mimics the neuronal effects of ghrelin in the arcuate nucleus and attenuates anorexia-cachexia syndrome in tumor-bearing rats. Am J Physiol Regul Integr Comp Physiol. 2016;311(1):R89-96.
42. Barrera JG, Jones KR, Herman JP, D'Alessio DA, Woods SC, Seeley RJ. Hyperphagia and increased fat accumulation in two models of chronic CNS glucagon-like peptide-1 loss of function. J Neurosci. 2011;31(10):3904-13.
43. Larsen PJ, Tang-Christensen M, Holst JJ, Orskov C. Distribution of glucagon-like peptide-1 and other preproglucagon-derived peptides in the rat hypothalamus and brainstem. Neuroscience. 1997;77(1):257-70.
44. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-8.
45. Zhao S, Kanoski SE, Yan J, Grill HJ, Hayes MR. Hindbrain leptin and glucagon-like-peptide-1 receptor signaling interact to suppress food intake in an additive manner. Int J Obes (Lond). 2012;36(12):1522-8.
46. Hayes MR, Bradley L, Grill HJ. Endogenous Hindbrain Glucagon-Like Peptide-1 Receptor Activation Contributes to the Control of Food Intake by Mediating Gastric Satiation Signaling. Endocrinology. 2009;150(6):2654-9.
47. Ruttimann EB, Arnold M, Hillebrand JJ, Geary N, Langhans W. Intrameal hepatic portal and intraperitoneal infusions of glucagon-like peptide-1 reduce spontaneous meal size in the rat via different mechanisms. Endocrinology. 2009;150(3):1174-81.
48. Krieger JP, Langhans W, Lee SJ. Vagal mediation of GLP-1's effects on food intake and glycemia. Physiol Behav. 2015;152(Pt B):372-80.

49. Punjabi M, Arnold M, Ruttimann E, Graber M, Geary N, Pacheco-Lopez G, Langhans W. Circulating glucagon-like peptide-1 (GLP-1) inhibits eating in male rats by acting in the hindbrain and without inducing avoidance. Endocrinology. 2014;155(5):1690-9.
50. Punjabi M, Arnold M, Geary N, Langhans W, Pacheco-Lopez G. Peripheral glucagon-like peptide-1 (GLP-1) and satiation. Physiol Behav. 2011;105(1):71-6.
51. Krieger JP, Arnold M, Pettersen KG, Lossel P, Langhans W, Lee SJ. Knockdown of GLP-1 Receptors in Vagal Afferents Affects Normal Food Intake and Glycemia. Diabetes. 2016;65(1):34-43.
52. Daniels D, Marshall A. Evaluating the potential for rostral diffusion in the cerebral ventricles using angiotensin II-induced drinking in rats. Brain Res. 2012;1486:62-7.
53. Price CJ, Hoyda TD, Ferguson AV. The area postrema: a brain monitor and integrator of systemic autonomic state. The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry. 2008;14(2):182-94.
54. Prasad-Reddy L, Isaacs D. A clinical review of GLP-1 receptor agonists: efficacy and safety in diabetes and beyond. Drugs in context. 2015;4:212283.
55. Tomas E, Stanojevic V, McManus K, Khatri A, Everill P, Bachovchin WW, Habener JF. GLP-1(32-36)amide Pentapeptide Increases Basal Energy Expenditure and Inhibits Weight Gain in Obese Mice. Diabetes. 2015;64(7):2409-19.
56. Choung JS, Lee YS, Jun HS. Exendin-4 increases oxygen consumption and thermogenic gene expression in muscle cells. Journal of molecular endocrinology. 2017;58(2):79-90.
57. Bernstein IL. Learned food aversions in the progression of cancer and its treatment. Ann N Y Acad Sci. 1985;443:365-80.
58. Jacobsen PB, Bovbjerg DH, Schwartz MD, Andrykowski MA, Futterman AD, Gilewski T, Norton L, Redd WH. Formation of food aversions in cancer patients receiving repeated infusions of chemotherapy. Behaviour research and therapy. 1993;31(8):739-48.
59. Bernstein IL, Fenner DP. Learned food aversions: heterogeneity of animal models of tumor-induced anorexia. Appetite. 1983;4(2):79-86.
60. Bernstein IL. Learned taste aversions in children receiving chemotherapy. Science. 1978;200(4347):1302-3.
61. Scalera G. Effects of conditioned food aversions on nutritional behavior in humans. Nutritional neuroscience. 2002;5(3):159-88.
62. Smith JC, Blumsack JT, Bilek FS, A.C. S, G.R. H, D.L. B. Radiation-induced taste aversion as a factor in cancer therapy. Cancer Treat Rep. 1984(68):9.
63. DeWys WD, Walters K. Abnormalities of taste sensation in cancer patients. Cancer. 1975;36(5):1888-96.
64. Hovan AJ, Williams PM, Stevenson-Moore P, Wahlin YB, Ohrn KE, Elting LS, Spijkervet FK, Brennan MT, Dysgeusia Section OCSGMAoSCiCISoOO. A systematic review of dysgeusia induced by cancer therapies. Supportive care in cancer : official journal of the Multinational Association of Supportive Care in Cancer. 2010;18(8):1081-7.
65. Hong JH, Omur-Ozbek P, Stanek BT, Dietrich AM, Duncan SE, Lee YW, Lesser G. Taste and odor abnormalities in cancer patients. The journal of supportive oncology. 2009;7(2):58-65.
66. Tsai VW, Manandhar R, Jorgensen SB, Lee-Ng KK, Zhang HP, Marquis CP, Jiang L, Husaini Y, Lin S, Sainsbury A, Sawchenko PE, Brown DA, Breit SN. The anorectic actions of the TGFbeta cytokine MIC-1/GDF15 require an intact brainstem area postrema and nucleus of the solitary tract. PloS one. 2014;9(6):e100370.

Fig. 1

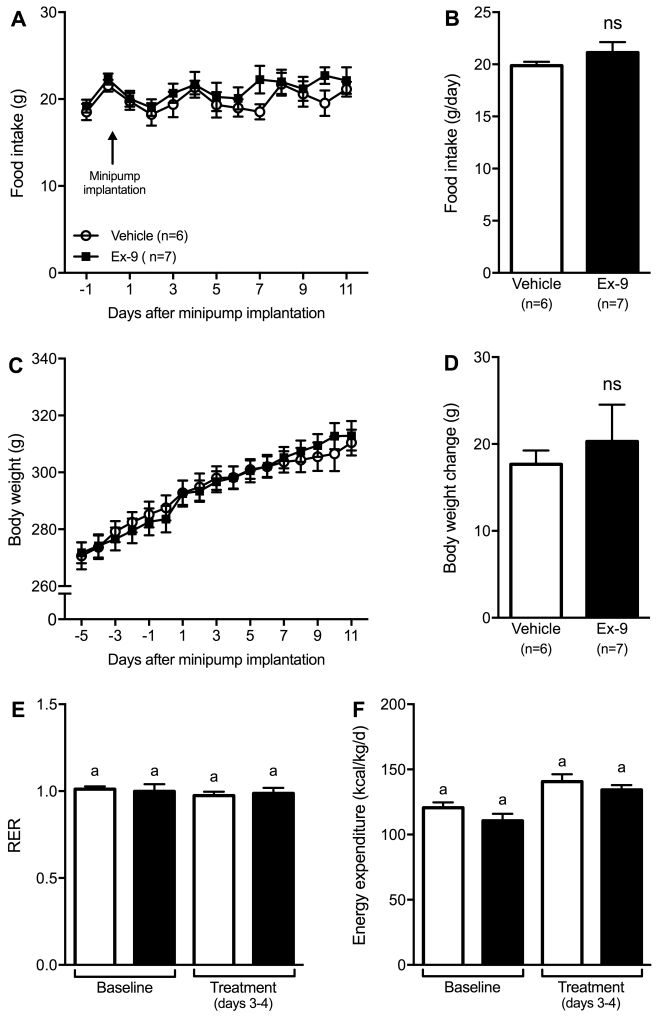

A
Food intake (g)
Minipump implantation
Vehicle (n=6)
Ex-9 (n=7)
Days after minipump implantation
B
Food intake (g/day)
ns
Vehicle (n=6)
Ex-9 (n=7)
C
Body weight (g)
Days after minipump implantation
D
Body weight change (g)
ns
Vehicle (n=6)
Ex-9 (n=7)
E
RER
a
Baseline
Treatment (days 3-4)
F
Energy expenditure (kcal/kg/d)
a
Baseline
Treatment (days 3-4)

Fig. 2

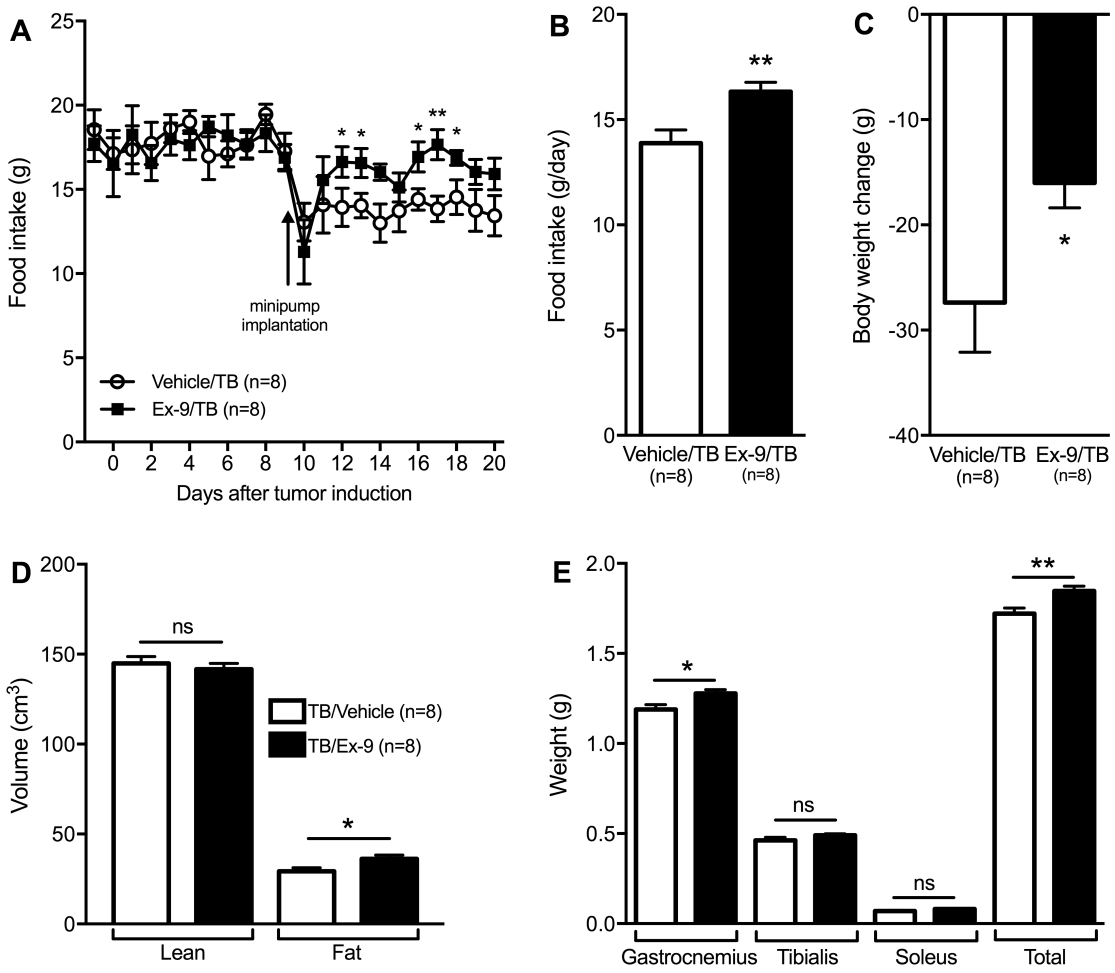

A
Food intake (g)
minipump implantation
Vehicle/TB (n=8)
Ex-9/TB (n=8)
Days after tumor induction
B
Food intake (g/day)
Vehicle/TB (n=8)
Ex-9/TB (n=8)
C
Body weight change (g)
Vehicle/TB (n=8)
Ex-9/TB (n=8)
D
Volume (cm3)
ns
TB/Vehicle (n=8)
TB/Ex-9 (n=8)
Lean
Fat
E
Weight (g)
Gastrocnemius
Tibialis
Soleus
Total

Fig. 3

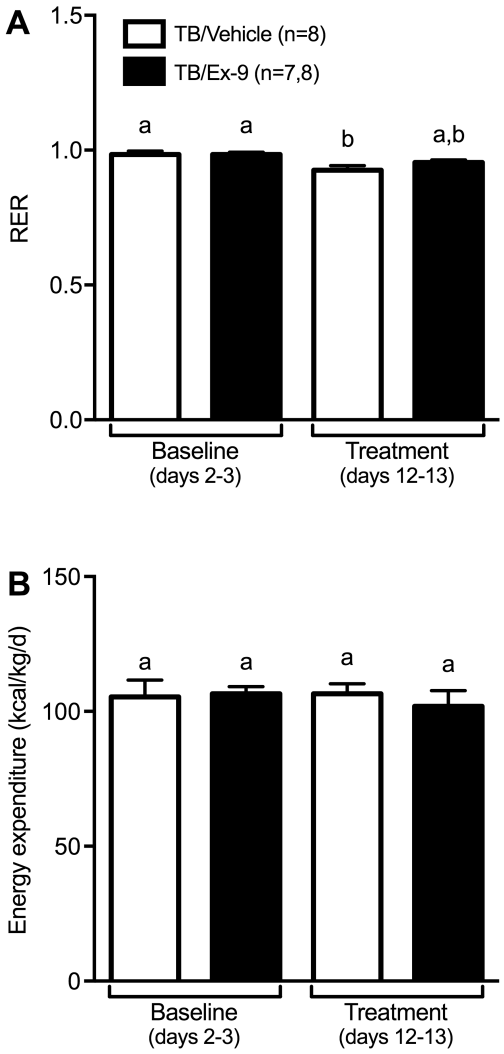

A
1.5
1.0
0.5
0.0
RER
TB/Vehicle (n=8)
TB/Ex-9 (n=7,8)
a
a
b
a,b
Baseline
(days 2-3)
Treatment
(days 12-13)
B
150
100
50
0
Energy expenditure (kcal/kg/d)
a
a
a
a
Baseline
(days 2-3)
Treatment
(days 12-13)

Fig. 4

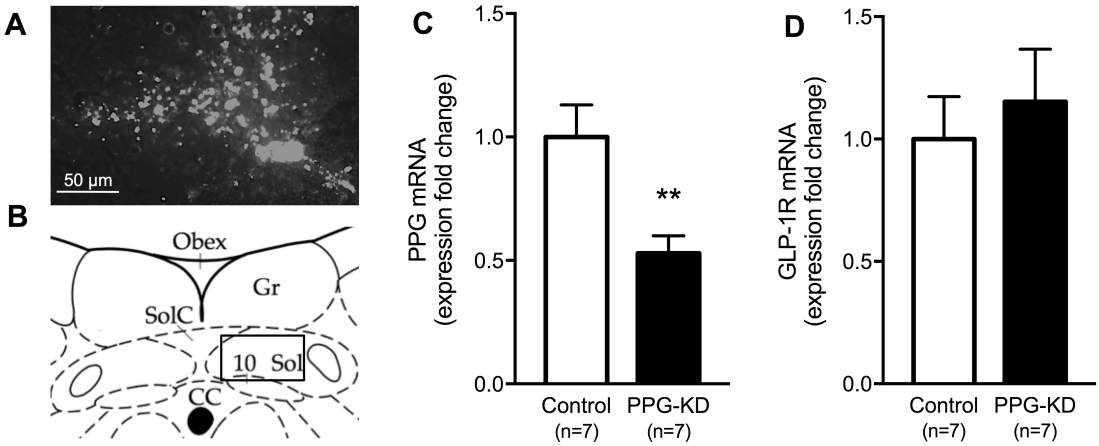

A
50 μm
B
Obex
Gr
SolC
10
Sol
CC
C
PPG mRNA
(expression fold change)
1.5
1.0
0.5
0.0
**
Control
(n=7)
PPG-KD
(n=7)
D
GLP-1R mRNA
(expression fold change)
1.5
1.0
0.5
0.0
Control
(n=7)
PPG-KD
(n=7)

Fig. 5

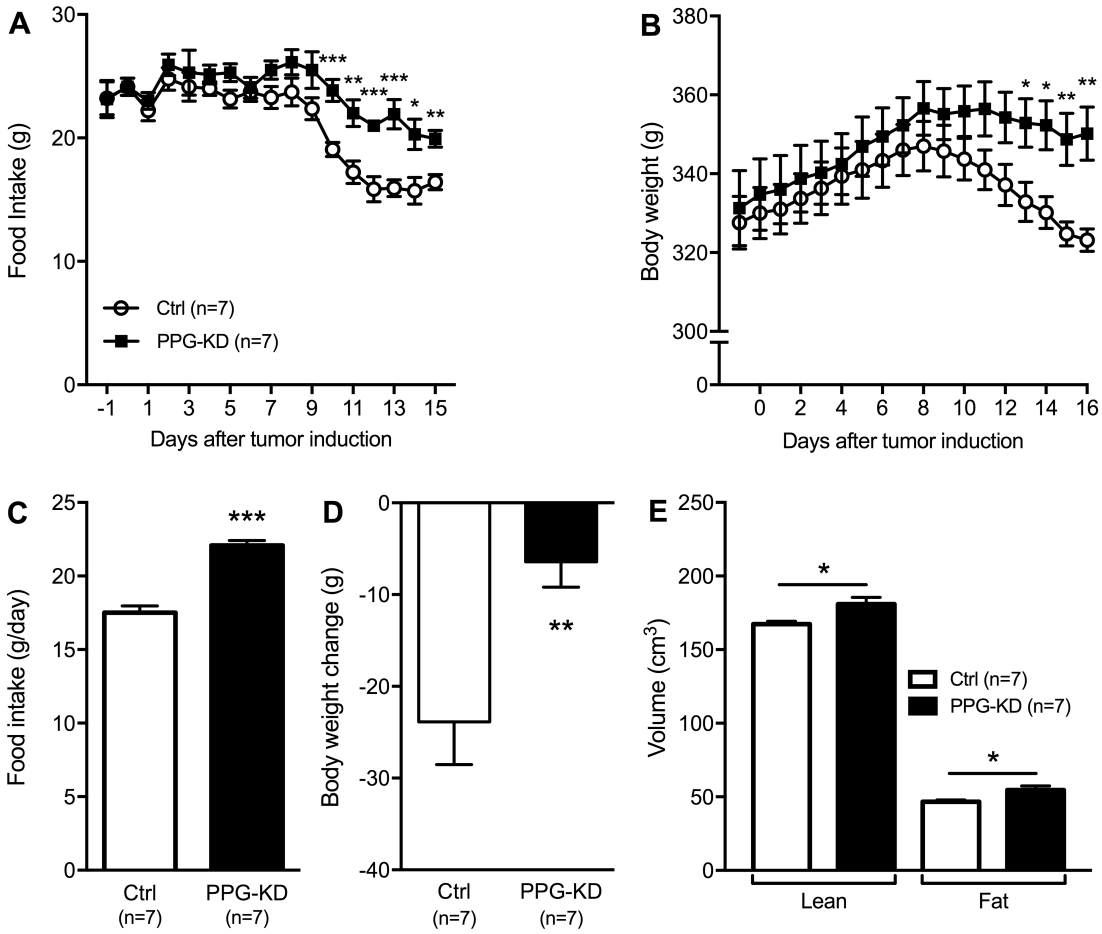

A
Food Intake (g)
Days after tumor induction
Ctrl (n=7)
PPG-KD (n=7)
B
Body weight (g)
Days after tumor induction
C
Food intake (g/day)
Ctrl (n=7)
PPG-KD (n=7)
D
Body weight change (g)
Ctrl (n=7)
PPG-KD (n=7)
E
Volume (cm3)
Ctrl (n=7)
PPG-KD (n=7)
Lean
Fat

Fig. 6

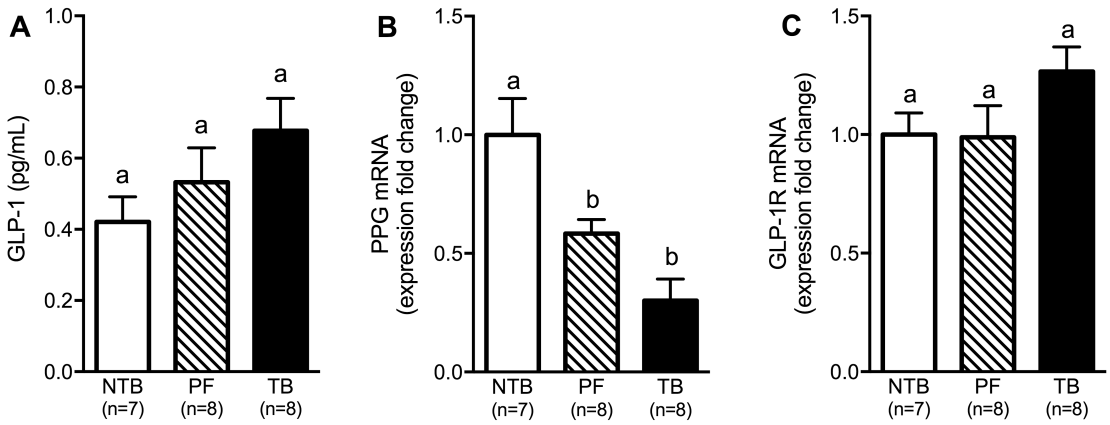

A
GLP-1 (pg/mL)
1.0
0.8
0.6
0.4
0.2
0.0
a
a
a
NTB (n=7)
PF (n=8)
TB (n=8)
B
PPG mRNA (expression fold change)
1.5
1.0
0.5
0.0
a
b
b
NTB (n=7)
PF (n=8)
TB (n=8)
C
GLP-1R mRNA (expression fold change)
1.5
1.0
0.5
0.0
a
a
a
NTB (n=7)
PF (n=8)
TB (n=8)

Fig. 7

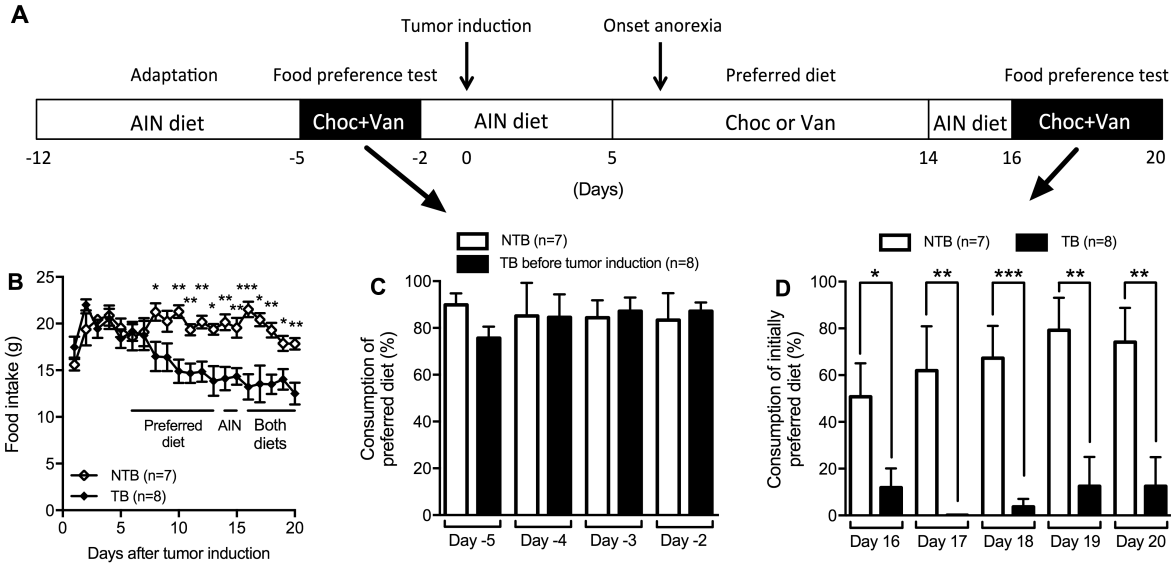
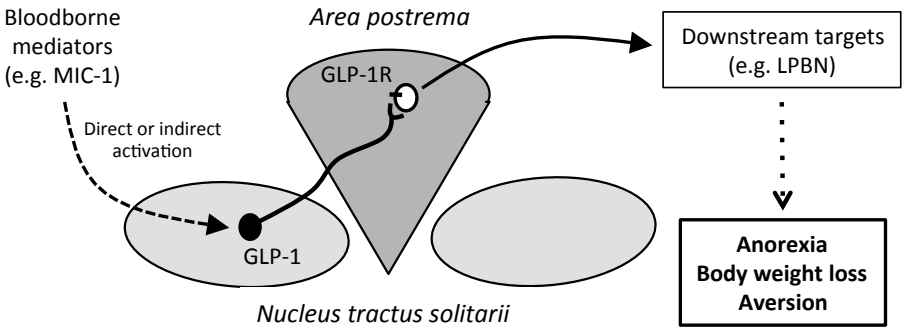

A
Tumor induction
Onset anorexia
Adaptation
Food preference test
Preferred diet
Food preference test
AIN diet
Choc+Van
AIN diet
Choc or Van
AIN diet
Choc+Van
-12
-5
-2
0
5
14
16
20
(Days)
B
Food intake (g)
Preferred diet
AIN
Both diets
NTB (n=7)
TB (n=8)
Days after tumor induction
C
NTB (n=7)
TB before tumor induction (n=8)
Consumption of preferred diet (%)
Day -5
Day -4
Day -3
Day -2
D
NTB (n=7)
TB (n=8)
Consumption of initially preferred diet (%)
Day 16
Day 17
Day 18
Day 19
Day 20

Fig. 8


Bloodborne
mediators
(e.g. MIC-1)
Direct or indirect
activation
Area postrema
GLP-1R
GLP-1
Nucleus tractus solitarii
Downstream targets
(e.g. LPBN)
Anorexia
Body weight loss
Aversion

7 Original Research Article: " The ghrelin receptor agonist HM01 mimics the neuronal effects of ghrelin in the arcuate nucleus and attenuates anorexia-cachexia syndrome in tumor-bearing rats"

The following section contains an original research article that was accepted for publication by the American Journal of Physiology: Regulatory, Integrative and Comparative Physiology in May 2016.

My contribution to this publication includes: conception and design of research, data acquisition, data analysis, data interpretation and writing the manuscript.

Am J Physiol Regul Integr Comp Physiol 311: R89–R96, 2016.
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The ghrelin receptor agonist HM01 mimics the neuronal effects of ghrelin in the arcuate nucleus and attenuates anorexia-cachexia syndrome in tumor-bearing rats

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Borner T, Loi L, Pietra C, Giuliano C, Lutz TA, Riediger T. The ghrelin receptor agonist HM01 mimics the neuronal effects of ghrelin in the arcuate nucleus and attenuates anorexia-cachexia syndrome in tumor-bearing rats. *Am J Physiol Regul Integr Comp Physiol* 311: R89–R96, 2016. First published May 4, 2016; doi:10.1152/ajpregu.00044.2016.—The gastric hormone ghrelin positively affects energy balance by increasing food intake and reducing energy expenditure. Ghrelin mimetics are a possible treatment against cancer anorexia-cachexia syndrome (CACS). This study aimed to characterize the action of the nonpeptidergic ghrelin receptor agonist HM01 on neuronal function, energy homeostasis and muscle mass in healthy rats and to evaluate its possible usefulness for the treatment of CACS in a rat tumor model. Using extracellular single-unit recordings, we tested whether HM01 mimics the effects of ghrelin on neuronal activity in the arcuate nucleus (Arc). Furthermore, we assessed the effect of chronic HM01 treatment on food intake (FI), body weight (BW), lean and fat volumes, and muscle mass in healthy rats. Using a hepatoma model, we investigated the possible beneficial effects of HM01 on tumor-induced anorexia, BW loss, muscle wasting, and metabolic rate. HM01 (10^{-7}–10^{-6} M) mimicked the effect of ghrelin (10^{-8} M) by increasing the firing rate in 76% of Arc neurons. HM01 delivered chronically for 12 days via osmotic minipumps (50 μg/h) increased FI in healthy rats by 24%, paralleled by increased BW, higher fat and lean volumes, and higher muscle mass. Tumor-bearing rats treated with HM01 had 30% higher FI than tumor-bearing controls and were protected against BW loss. HM01 treatment resulted in higher muscle mass and fat mass. Moreover, tumor-bearing rats reduced their metabolic rate following HM01 treatment. Our studies substantiate the possible therapeutic usefulness of ghrelin receptor agonists like HM01 for the treatment of CACS and possibly other forms of disease-related anorexia and cachexia.

cancer; malnutrition; food intake; metabolism; hypothalamus

THE CANCER ANOREXIA-CACHEXIA syndrome (CACS) is characterized by reduced eating, increased catabolism, and body weight loss. CACS is a major cause for the decline in the clinical status of affected patients and is associated with increased mortality. CACS is present in up to 80% of cancer patients at death and represents the most significant negative predictor of treatment outcome (12, 23, 41). Affected cancer patients show a poor responsiveness to anticancer therapies, irrespective of the type of malignancy (1, 9). The effectiveness of nutritional care as a therapeutic option against CACS is limited, and insufficient progress has been made in the development of specific pharmacological approaches (21).

Because of positive effects on energy balance, the gastrointestinal hormone ghrelin or ghrelin analogs are considered as a possible treatment option for CACS. Ghrelin is mainly secreted from the stomach, and it has been identified as a high-affinity ligand for the growth hormone secretagogue receptor (GHS-R) (20). Plasma levels of ghrelin rise during fasting and shortly before meals. Pharmacological doses of ghrelin stimulate food intake and promote body weight gain in rodents (36, 42). These effects are thought to be mediated via the hypothalamic arcuate nucleus (Arc), which is of high importance for the control of food intake and energy homeostasis (32). The GHS-R is highly expressed in the Arc and ghrelin's effects on neuronal activity of Arc neurons have been characterized in various electrophysiological and immunohistological studies (13, 16, 17). Ghrelin activates neuropeptide Y-expressing neurons in the Arc, which is widely considered as the neuronal correlate for ghrelin's actions on food intake and body weight (5, 34, 45). Moreover, ghrelin also promotes muscle cell differentiation in vitro and ameliorates skeletal muscle atrophy in mice, suggesting that ghrelin may also exert direct effects on muscle that are independent of its central actions (10, 31).

Ghrelin has been tested as anti-CACS treatment in clinical human trials and rodent cancer models (4, 8, 15, 25–27, 39, 46). Most of these studies support a beneficial effect of ghrelin on food intake and body weight. However, because of its peptidergic nature and its short half-life time of 15–20 min, the usefulness of native ghrelin as a therapeutic agent is limited (44). HM01 is a synthetic small-molecule compound that acts as a GHS-R agonist. It has high receptor binding affinity, high brain permeability, and a higher plasma half-life compared with ghrelin (19). Besides other possible therapeutic indications, it might, therefore, be used as an anti-CACS treatment. It was the overall aim of our studies to evaluate the possible usefulness of HM01 against tumor-dependent anorexia and body weight loss. First, we sought to confirm the ghrelin-like excitatory action of HM01 in electrophysiological recordings of the Arc of rats. Second, we investigated the effect of chronic HM01 treatment on food intake, body weight, muscle mass, and body composition in healthy non-tumor-bearing rats. Third, we used a rat Morris-7777 hepatoma model to test whether HM01 ameliorates tumor anorexia, body weight loss, and muscle wasting, and whether HM01 affects nutrient utili-

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zation and metabolic rate. The Morris-7777 hepatoma model is well established and characterized by a clear anorectic response and body weight loss (35). Since an attenuation of anorexia is one important mode of action for ghrelin-based approaches, we chose a tumor model in which anorexia is a major contributor to body weight loss. Another advantage of this tumor model is that anorexia and body weight loss are less severe than in other tumor models. A very rapid deterioration of the health status caused by aggressive tumors often limits the usefulness of such tumor models for chronic treatment paradigms.

METHODS

Animals. Adult male Wistar rats (Elevage Janvier, France), weighing between 230 and 270 g, were used for all electrophysiological and behavioral experiments involving non-tumor-bearing animals. Animals were kept in a temperature-controlled room (21 ± 1°C) on a 12:12-h light-dark cycle with ad libitum access to standard chow (890 25 W16, Provimi Kliba) and tap water. For the in vivo testing of the GHS-R agonist HM01, rats were single-housed in wire-mesh cages. Before the experiments, rats were handled daily and kept in the cages for an adaptation period of at least 1 wk.

For the behavioral and metabolic studies involving tumor-bearing animals, adult male Buffalo rats (Charles River) weighing around 280 g were used. The animals were single-housed in metabolic cages (TSE Phenomaster, TSE Systems) and were adapted to the housing conditions for 7 days before the start of the experiment. To evaluate the effects of tumor growth on muscle mass, Buffalo rats were single-housed in wire-mesh cages. All animal procedures were approved by the Veterinary Office of the Canton of Zurich, Switzerland.

Electrophysiology. The electrophysiological recording technique was the same as described previously (3). The rats were decapitated using a guillotine at the same time point in the middle of the light phase to minimize differences in the circadian and prandial state of the animals. After decapitation, the brain was quickly removed and superfused with ice-cold artificial cerebrospinal fluid (aCSF) that was oxygenated and pH-equilibrated with oxycarbon (pH 7.4; 290 mosmol/kg H_2O). Coronal brain slices (700 μm) were cut at the mid-rostro-caudal level of the Arc using a vibratome (Leica VT1000S, Leica Microsystems). A rectangular 3 × 3-mm slice preparation containing the Arc was manually dissected under a dissection microscope and transferred to a temperature-controlled (37°C) incubation chamber filled with constantly oxygenated aCSF. For recordings, the Arc preparations were transferred to a temperature-controlled (37°C) recording chamber that was constantly perfused with oxygenated and prewarmed aCSF at a rate of 1.6 ml/min. Extracellular single-unit recordings were obtained using home-made glass-coated platinum-iridium electrodes. Recordings were conducted in the medial arcuate nucleus, in which the majority of neurons are excited by ghrelin (34). HM01 (provided by Helsinn Healthcare, Lugano, Switzerland) was superfused at a concentration of 10^{-6} or 10^{-7} M. The cosensitivity of the recorded neurons to ghrelin was tested by stimulation with rat ghrelin (10^{-8} M; Bachem).

Effects of HM01 in non-tumor-bearing rats. Osmotic minipumps (model 2ML2, ALZET osmotic pumps; DURECT) were implanted subcutaneously into the left flank at the lower level of the abdomen between the chest and the hind limbs. Minipumps were filled with HM01 (10 μg/μl) or saline for controls. The minipumps released a constant amount of HM01 (50 μg/h at a pump rate of 5 μl/h). The capacity of the minipumps (2 ml) allowed a constant compound release for 14 days. Body weight and food intake were measured daily. At the end of the experiment, animals were euthanized for the measurement of body composition by computed tomography (CT) scanning and muscle mass.

Effects of HM01 in tumor-bearing rats. The hepatoma tumor model was used in our experiments, as previously described (35). Morris hepatoma 7777 cells (McA-RH7777, cat. no. CRL-1601, American Type Culture Collection) were cultured under standard conditions in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Semiconfluent McA-RH7777 Petri dishes were washed with DMEM repeatedly to detach the cells from the surface. After confirming vitality of the cells with Trypan blue, 10^7 cells were inoculated subcutaneously in 250 μl PBS under short isoflurane anesthesia between the scapulas.

HM01 or saline treatment was applied as described above. Minipumps were implanted on *day 11* after tumor inoculation, shortly after the onset of the anorectic response. Treatments continued for 6 days until the animals had to be euthanized for ethical reasons, according to our criteria for the termination of experiments. Food intake and respiratory gas exchange (O_2 and CO_2) were recorded automatically at 17-min intervals throughout the entire experiment. Body weight was measured daily. After euthanizing the rats, tumors and limb muscles were resected and weighed, and body composition was determined by CT scan (see *Computed tomography scanning and muscle weight measurements*).

To examine the effects of tumor growth on muscle mass, tumor-bearing and non-tumor-bearing rats were used. After adaptation, tumor growth was induced as described above; control animals received the same volume of PBS without tumor cells. Rats were euthanized 16 days after tumor induction, and limb muscles were dissected and weighed.

Computed tomography scanning and muscle weight measurements. Total carcass lean and fat volumes were measured by quantitative microcomputed tomography (La Theta LCT-100A scanner, Hitachi-Aloka Medical). Sequential 2-mm slice images with a pixel size of

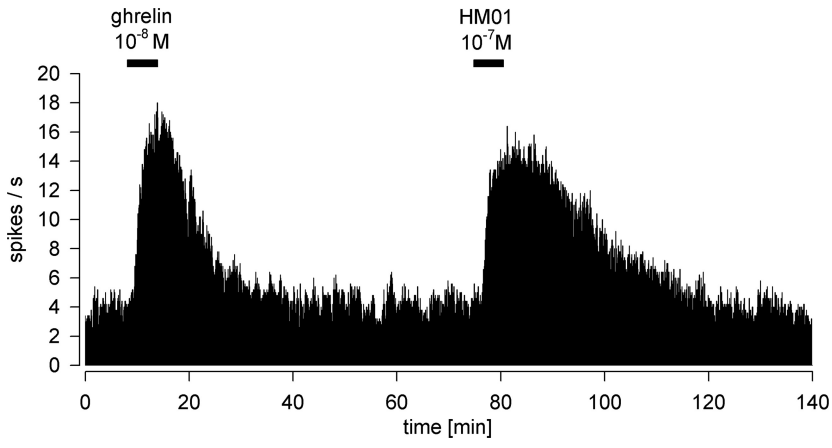

Fig. 1. Recording of a ghrelin-excited Arc neuron, which was also reversibly stimulated by superfusion of HM01. Horizontal bars indicate time of superfusion.

Table 1. *Effect parameters of the excitatory responses after superfusion of ghrelin (10^{-8} M), HM01 10^{-6} M, and HM01 10^{-7} M*

Parameters	Ghrelin 10^{-8} M (n = 13)	HM01 10^{-6} M (n = 8)	HM01 10^{-7} M (n = 5)
Mean spontaneous activity, Hz	2.7 ± 0.5	2.5 ± 0.7	1.7 ± 0.7
Mean latency, s	53 ± 7.9[a]	63 ± 13.7[a]	128 ± 36.3[b]
Absolute response, Hz	2.5 ± 0.6	2.7 ± 0.6	3.9 ± 1.1
Absolute peak response, Hz	5.2 ± 1	4.5 ± 1	6.1 ± 1.7
Mean response duration, s	899 ± 76.2[a]	2103 ± 552[b]	1952 ± 537[a]

Values are expressed as means ± SE. Data were analyzed with one-way ANOVA followed by Tukey post hoc test. Means with different letters are significantly different.

250 × 250 μm were used for calculations using LaTheta software (version 2.10). Head, lungs, and tail were excluded from the analysis of tissue volumes. After the CT scanning, the left gastrocnemius, tibialis, and soleus muscles were dissected at the level of their upper to lower tendons and were weighed.

Quantification and statistical analysis. Electrophysiological parameters were quantified as previously described (3). The mean spontaneous baseline activity of each single neuron was evaluated for 60 s before the stimulus (spontaneous activity). This value was used to normalize changes in the firing rate expressed as absolute mean response change (Hz) and as a percentage of mean response change. If both the absolute and the percentage of discharge rate changes during the response were larger than ± 0.5 Hz and ± 20%, respectively, the neuron was considered sensitive to the applied substance. Furthermore, the latency, the duration, and the peak response (Hz) were also measured. The effect parameters of the electrophysiological responses were averaged and expressed as means ± SE. Values were compared using one-way ANOVA followed by Tukey's post hoc test.

For the behavioral experiments, daily and cumulative food intake, body weight, and body composition volumes were expressed as means ± SE. Body weight change following treatment was calculated by subtracting the body weight at the time of minipump implantation from the body weight at the end of the treatment period. These values were corrected for the weight of the minipumps and the tumor mass that was determined by tumor resection. Data were expressed as means ± SE and statistically compared using Student's t-test (two-sided).

In tumor-bearing rats, the respiratory exchange ratio (RER) and energy expenditure (EE) were calculated from O_2 consumption and CO_2 production, as described previously (33). EE data were normalized for body weight. The calculation was based on the following equation: total EE (kcal·kg^{-1}·h^{-1}) = (3.9 × Vo_2 + 1.1 × Vco_2)/1,000; RER = Vco_2 / Vo_2. The average of *days 1* and *2* after tumor inoculation was used as baseline (i.e., prior to the onset of anorexia) and compared with the values obtained on *days 14* and *15* during the treatment period (i.e., during the anorectic phase). Group means were analyzed with one-way ANOVA followed by the Tukey post hoc test. After tumor resection, tumor weights were compared between control animals and HM01-treated rats by Student's t-test (two-sided). For all statistical analyses, $P < 0.05$ was considered significant.

RESULTS

Electrophysiology. Seventeen Arc neurons were recorded. Similar to previous studies, the majority (13/17; 76%) of neurons recorded in the medial Arc was reversibly excited by ghrelin (10^{-8} M) (34). All ghrelin-excited cells were also activated by superfusion of HM01 at a concentration of 10^{-7}–10^{-6} M (see example in Fig. 1). Interestingly, neurons that were insensitive (3/17; 18%) or inhibited (1/17; 6%) by ghrelin showed the same type of responses to HM01, resulting in 100% concordant responses. The average response latency was significantly longer for the lower concentration of HM01 (10^{-7} M) compared with ghrelin or HM01 at 10^{-6} M. The absolute changes in firing rate and the peak responses were not significantly different between the stimuli. However, HM01 tended to induce longer-lasting responses compared with ghrelin, which reached statistical significance for the higher concentration of HM01 (Table 1).

Effects of HM01 in non-tumor-bearing rats. HM01 significantly stimulated food intake starting on the first day after minipump implantation. This effect remained stable during the 12 days of measurements. On average HM01-treated rats consumed 6.0 ± 0.3 g (24 ± 1%) more food per day than controls (Fig. 2*A*). Over the 12-day treatment period, this resulted in a significantly higher cumulative food intake (HM01, 405 ± 6 g; saline, 329 ± 7 g; $P < 0.001$). This orexigenic effect of HM01 was paralleled by an increase in body weight relative to controls, which became significant on *day 5* after minipump implantation (Fig. 2*B*). At the end of the experiments, the difference in body weight was 35 g

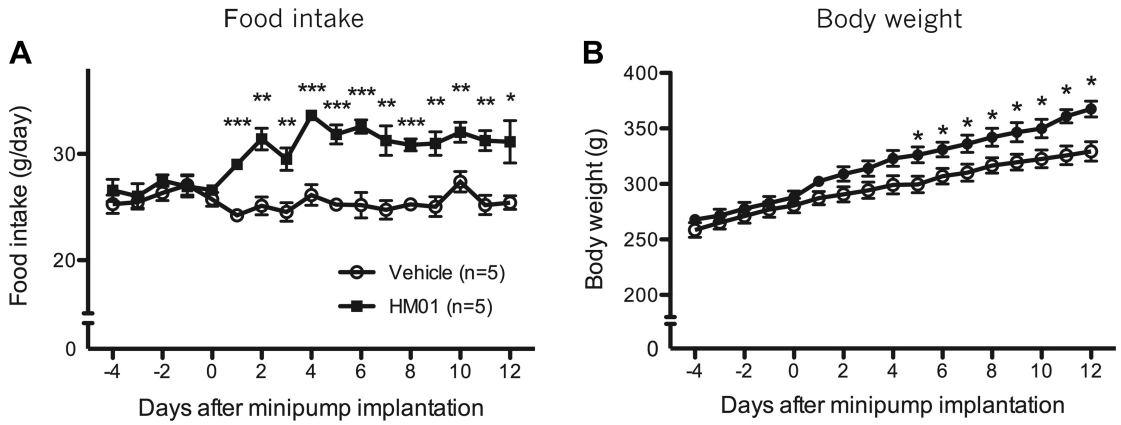

Fig. 2. Daily food intake and body weight of HM01-treated (50 μg/h) and vehicle-treated animals. *A*: HM01-stimulated food intake starting from the day after minipump implantation. This effect remained stable during the 12-day treatment period. *B*: HM01-induced increase in food intake was paralleled by an increase in body weight relative to controls, which became significant from *day 5* after minipump implantation. Data analyzed using the Student's t-test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

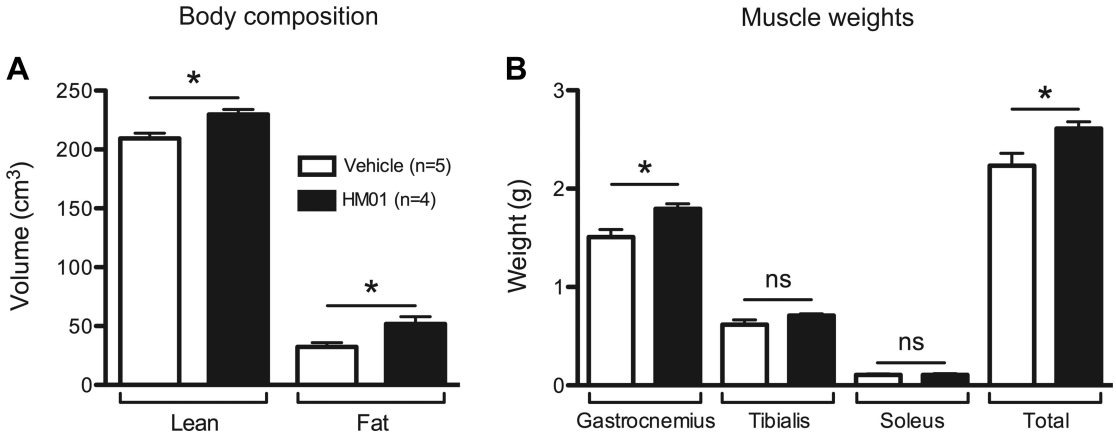

Fig. 3. Computed tomography-based assessment of lean and total fat volumes, and weights of gastrocnemius, tibialis, and soleus muscle. *A*: rats treated with HM01 (50 μg/h) displayed higher lean and fat volumes than vehicle-treated animals. *B*: HM01 treatment also resulted in higher gastrocnemius and total muscle mass (sum of gastrocnemius, tibialis, and soleus mass) compared with controls. Data were analyzed using the Student's *t*-test (*$P < 0.05$; ns, not significant).

(369 ± 6 g vs. 334 ± 8 g; $P < 0.05$). HM01 significantly increased lean and fat body mass after the 12-day treatment period (229.7 ± 4.3 vs. 209.5 ± 4.3; $P < 0.05$ and 51.6 ± 6.1 cm^3; vs. 32.2 ± 3.5 cm^3; $P < 0.05$; respectively, Fig. 3*A*). Moreover, rats treated with HM01 had a higher gastrocnemius weight compared with saline controls (HM01: 1.795 ± 0.052 g vs. control: 1.508 ± 0.076 g; $P < 0.05$, Fig. 3*B*).

Effects of HM01 in tumor-bearing rats. In tumor-bearing animals food intake started to decline around *day 7* after tumor induction. After implantation of minipumps on *day 11*, HM01 prevented a further decrease in eating, and HM01-treated rats consumed significantly more food than control animals (Fig. 4*A*). On average, daily food intake during the treatment period (i.e., from *day 12* to *day 17* after tumor induction) was 18.5 ± 0.6 g vs. 14.3 ± 0.5 g (saline controls), which corresponds to a 30% higher food intake of HM01-treated rats compared with controls (Fig. 4*B*). While tumor-bearing controls showed a loss of body weight during the treatment period, rats receiving HM01 stabilized their body weight. The difference in absolute body weight did not reach statistical significance; however, the change in body weight was highly significant between the treatment groups (HM01: 1.1 ± 2.0 g vs. control: −10.4 ± 1.7 g; $P < 0.001$; Fig. 4*C*). This difference in body weight change appeared to be mainly due to lower fat mass in tumor-bearing control rats because HM01-treated rats showed significantly higher fat mass but similar lean mass compared with control animals (Fig. 5*A*). Tumor-bearing rats had lower gastrocnemius, tibialis, and soleus muscle mass compared with controls (1.077 ± 0.018 vs. 1.208 ± 0.038, 0.342 ± 0.012 vs. 0.397 ± 0.022, 0.059 ± 0.002 g vs. 0.067 ± 0.001 g; $P < 0.05$; in all cases, Fig. 5*B*). HM01 led to a higher gastrocnemius and soleus mass compared with tumor-bearing controls (1.422 ± 0.050 vs. 1.253 ± 0.054, 0.091 ± 0.005 g vs. 0.072 ± 0.005 g; $P < 0.05$; respectively, Fig. 5*C*).

Relative to baseline conditions before the onset of anorexia (*days 1* and *2*), control rats had significantly lower RER values on *days 14* and *15* reflecting a shift toward lipid metabolism. This tumor-dependent reduction in RER was partly prevented by HM01 treatment (Fig. 6*A*). Interestingly, energy expenditure was significantly lower in HM01-treated compared with control rats during the treatment period (*days 14* and *15*) and also lower relative to baseline conditions on *days 1* and *2* (Fig. 6*B*). Chronic HM01 treatment did not affect tumor growth

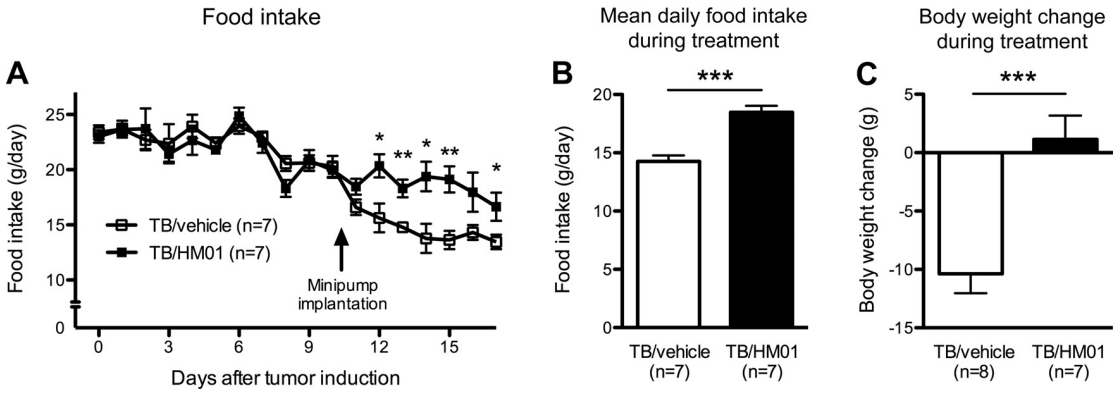

Fig. 4. Daily food intake, mean daily food intake during treatment, and body weight development of HM01-treated (50 μg/h) and vehicle-treated tumor-bearing (TB) rats following minipump implantation. *A*: HM01 significantly attenuated the anorectic response induced by tumor growth. *B*: on average, HM01-treated TB rats had a 30% higher daily food intake compared with TB controls. *C*: chronic HM01 administration prevented tumor-induced body weight loss. Data analyzed using the Student's *t*-test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

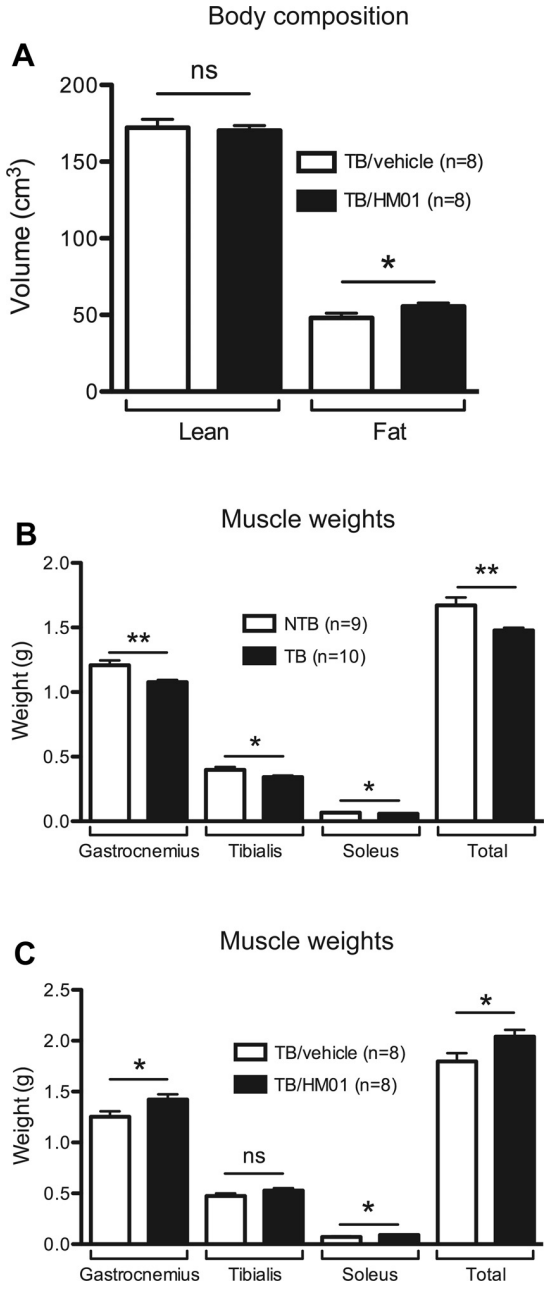

Fig. 5. Computed tomography-based assessment of lean and total fat volumes and limb muscle weights. *A*: compared with tumor-bearing (TB) control animals, HM01-treated rats (50 μg/h,) had higher fat volumes. *B*: tumor growth was associated with reduced gastrocnemius, tibialis, soleus, and total muscle mass (sum of gastrocnemius, tibialis, and soleus muscle mass), suggesting the development of tumor-induced muscle atrophy (NTB: non-tumor-bearing controls). *C*: HM01 treatment led to higher gastrocnemius, soleus, and total muscle mass compared with tumor-bearing controls. Data analyzed using the Student's *t*-test (*$P < 0.05$, **$P < 0.01$).

because average tumor weight was not significantly different between treatment groups (8.9 ± 1.0 g vs. 8.2 ± 0.7 g, Fig. 7).

DISCUSSION

We provide in vitro and in vivo characterization of the synthetic GHS-R agonist HM01 under nonpathological conditions and in the context of cancer anorexia and body weight loss. In particular, we showed that HM01 acts as a ghrelin analog in the Arc, increases food intake and body weight in healthy rats, and prevents the tumor-induced body weight loss by attenuating anorexia and by reducing energy expenditure. In the electrophysiological experiments, HM01 and ghrelin showed a concordant response profile in all tested neurons. Hence, HM01 mimicked the effects of ghrelin on neuronal activity of Arc neurons.

We performed recordings using two concentrations of HM01 (10^{-7} M and 10^{-6} M), and we observed a significantly longer response duration after superfusion of the higher concentration of HM01 (10^{-6} M) compared with ghrelin. However, no difference was seen in the absolute response and the peak response. It was not the aim of this study to establish a complete dose-response characteristic for the excitatory effects of HM01, but only to provide proof of concept that HM01 and ghrelin exert concordant actions on Arc neurons. To use effective stimuli, we used concentrations that are clearly above the EC_{50} of 1–2 nM for HM01 (19). Therefore, a possible ceiling effect might be the reason why the absolute and peak responses caused by the two concentrations were similar. We cannot exclude differences in the dynamics of receptor activation by ghrelin and HM01, respectively, which might be reflected by the longer latency of HM01 (10^{-7} M) compared with ghrelin (10^{-8} M). In addition to the direct excitatory effect of ghrelin on NPY neurons, ghrelin indirectly inhibits POMC neurons in the Arc (5). It was not our aim to characterize the effect of HM01 on second-order neurons. Nevertheless, an indirect inhibition of POMC neurons is a likely downstream effect of HM01.

The ghrelin-like action of HM01 on neuronal activity in the Arc is in line with the orexigenic effect of HM01 observed in the current study. HM01 treatment in healthy rats was associated with increased body weight gain and increased lean and fat mass. These effects were similar to the action induced by chronic treatment with the ghrelin analog BIM-28131, which has been considered as one of the most potent GHS-R agonists (28, 29, 38). Interestingly, we observed an increase in both absolute fat and lean volumes and in gastrocnemius muscle mass in HM01-treated rats. While most studies using ghrelin or GHS-R agonists demonstrate increased adiposity in healthy rodents, treatment effects on lean and muscle mass appear to be less robust (22, 24, 38, 42). Ghrelin or BIM-28131-induced positive effects on lean body mass in healthy rats, although most of these effects only occurred after longer treatment periods compared with the current study (4, 28, 29, 38).

On the basis of previous studies, a possible feeding-independent adipogenic action of HM01 could be due to an upregulation of lipogenic enzymes and a reduction of lipid export in adipose tissue (7, 30). Another factor promoting adiposity may be a reduction of metabolic rate, which is a well-documented effect of ghrelin. This effect is mediated via reduced sympathetic outflow to brown adipose tissue that leads to decreased uncoupling protein-1 expression (43, 48). It was beyond the scope of our study to reconfirm the aforementioned mechanisms. On the basis of the similarity between the action of ghrelin and HM01, it appears plausible that similar adipogenic and metabolic processes might also be engaged by HM01.

Similar to its orexigenic action, ghrelin's effect on GH release depends on the GHS-R, although both effects occur

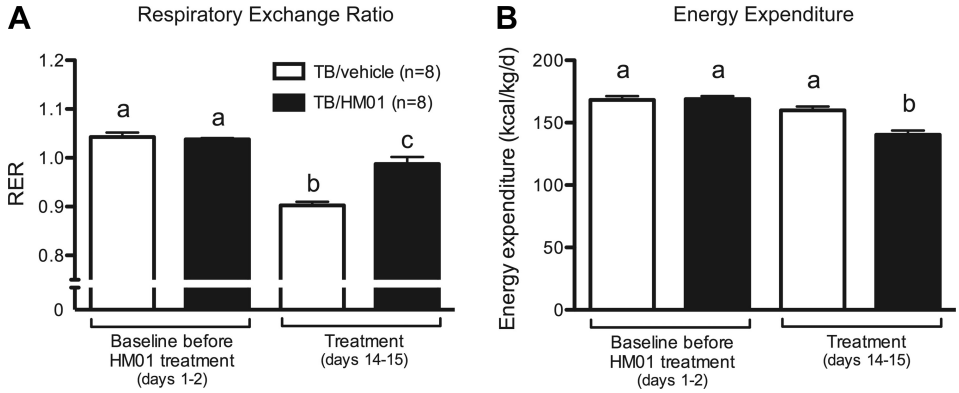

Fig. 6. Respiratory exchange ratio and energy expenditure of HM01-treated (50 μg/h) and vehicle-treated tumor-bearing (TB) rats. *A*: during treatment, TB control rats had significantly lower RER values compared with baseline conditions, reflecting a shift toward lipid metabolism. HM01 treatment prevented this tumor-induced reduction in RER. *B*: energy expenditure of the tumor-bearing HM01-treated group was significantly lower compared with the control group and to baseline conditions. Data analyzed using the one-way ANOVA followed by the Tukey post hoc test. Bars with different letters are significantly different, $P < 0.05$.

independently (40). Hence, the effect of HM01 on lean body mass might at least be partly mediated by HM01-dependent growth hormone/insulin-like growth factor 1 (GH/IGF-1) signaling (20). IGF-1 is a well-known mediator of GH-dependent muscle growth acting via the Akt protein kinase pathway to promote muscle protein synthesis (37). HM01 is a potent stimulator of GH release (Pietra C, unpublished findings). Therefore, a stimulation of GH/IGF-1 signaling is likely to contribute to the HM01-dependent increase in lean mass.

A major aim of our study was to evaluate the ability of HM01 to counteract tumor anorexia and body weight loss. In line with its potent action on food intake in healthy non-tumor-bearing animals, HM01 prevented the progression of the anorectic response and the loss of body weight in tumor-bearing rats. Moreover, HM01 reduced metabolic rate, lipid metabolism, and muscle wasting.

The relative increase in food intake of HM01-treated animals was similar in tumor-bearing and in non-tumor-bearing rats (30% and 24%, respectively). The magnitude of the positive effects of HM01 on food intake and body weight in tumor-bearing animals was also similar to the actions of ghrelin analogs used in previous studies (8). However, direct comparisons between the effectiveness of HM01 and ghrelin analogs used in other studies are limited due to different tumor models and treatment paradigms, as well as potential differences in dose-response relationships and pharmacokinetics of the substances used.

Hepatoma tumor-bearing rats had lower muscle mass compared with non-tumor-bearing controls, indicating tumor-induced muscle degradation. HM01 not only stimulated muscle growth in healthy animals, but it also increased muscle mass in tumor-bearing rats. Muscle atrophy during cancer is associated with decreased expression of different markers for muscle formation such as myoD, myogenin, and mTOR, and increased proteolytic markers like myostatin, activin A, FOXO, MURF-1, and atrogin-1/MAFbx (two muscle-specific ubiquitin ligases) (2). Chronic ghrelin treatment prevented the cancer-induced increase of myostatin, MURF-1, and atrogin-1/MAFbx in Lewis lung carcinoma-bearing mice. In parallel, it increased myoD and myogenin signaling (4). Whether similar mechanisms contribute to the HM01-mediated attenuation of cancer-induced muscle loss in our hepatoma tumor model remains to be elucidated.

Although we did not measure locomotor activity in the present study, we did not detect a tumor-dependent alteration of physical activity in hepatoma tumor-bearing rats in previous experiments. Hence, reduced physical activity does not appear to be a major determinant of muscle mass loss under our experimental conditions. Although our studies were not designed to test the influence of activity on muscle mass, it appears plausible that enhanced physical activity might further improve the therapeutic action of HM01.

Notably, HM01 treatment reduced energy expenditure. This is the first study showing a beneficial effect of a ghrelin-based therapy on energy expenditure during CACS. Therefore, not only the higher food consumption induced by HM01 but also the reduction in energy expenditure might have contributed to the preservation of body weight under our conditions. The reduction of RER following tumor induction reflects a shift from glucose to increased fat utilization. Chronic administration of HM01 attenuated the suppressive effect of tumor growth on RER, which indicates decreased fat metabolism in HM01-treated rats compared with controls. The latter effect is consistent with the preservation of fat depots by HM01 treatment.

Although an activation of GH/IGF-1 axis might have beneficial effects on lean and fat mass, it also raises concerns of potentially stimulating tumor proliferation. Nevertheless, experiments conducted in different rodent models and clinical studies have so far demonstrated the safety of ghrelin-based therapeutic approaches without indications of increased tumor growth or cancer progression (8, 15, 25, 27, 39, 46). Our

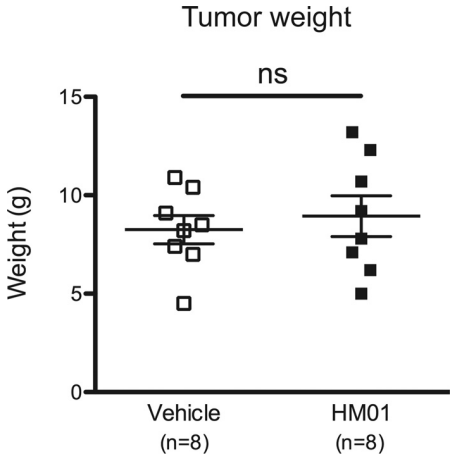

Fig. 7. Tumor weight of HM01-treated and control rats at the end of the 12-day treatment period. Tumor weight did not differ between the two groups, indicating that chronic HM01 treatment did not affect tumor growth.

observation that HM01 does not promote tumor growth is consistent with these findings. The lack of difference in the tumor weight between the HM01-treated animals and the control group is an important finding that is relevant for future long-term clinical applications.

In the context of CACS, the role of endogenous ghrelin is controversial. Ghrelin has been found increased, unchanged, or decreased in different rodent tumor models and among patients with different types of cancers (6, 11, 14, 18). We did not detect any changes in total or active ghrelin circulating level in our animals at the end of experiment (data not shown). Hence, a reduction in endogenous ghrelin levels does not appear to mediate the observed reduction in food intake in this rat tumor model.

Some studies postulated that CACS could lead to partial ghrelin resistance, which might eventually limit the effectiveness of ghrelin-based therapy (11, 47). This might be the reason why some ghrelin-based clinical trials have failed to attenuate CACS (39). However, under our experimental conditions, the antianorectic action of HM01 and its positive effect on body weight were still significant at the end of the treatment period. Moreover, the relative difference in food intake between HM01-treated rats and controls was similar in tumor-bearing and non-tumor-bearing rats, suggesting the absence of profound ghrelin resistance. In our studies, the effect of HM01 on food intake became more variable toward the end of the experiment, that is, just before our criteria for the termination of the experiment were reached. It is a general and expected phenomenon that the increasing tumor burden overrides anti-CACS treatment effects at late disease stages in experimental cancer models (15). Notably, an important purpose of anti-CACS treatments is to positively influence the health status and the anti-cancer treatment success before the patients undergo end-stage disease. For these reasons, we did not attempt to characterize the effectiveness of HM01 to counteract CACS at later time points.

In addition to the direct effect of HM01 on energy homeostasis, there might be beneficial effects resulting from anti-inflammatory actions. Ghrelin has been shown to attenuate the inflammatory cytokine response in tumor-bearing mice (4). In contrast to other tumor models, the possible inflammatory or cytokine-dependent mechanisms that contribute to CACS in our hepatoma tumor model have not yet been identified. Systemic levels of IL-1β, IFN-γ, IL-6, and TNF-α have been reported to be unaltered in this tumor model (35). Although this does not exclude that other cytokines contribute to CACS in hepatoma tumor-bearing rats, other tumor models appear to be more appropriate to investigate anti-inflammatory effects of HM01 (4). An attenuation of the inflammatory response might not only indirectly attenuate anorexia, but also cytokine-mediated effects on muscle wasting.

Perspectives and Significance

We demonstrated that HM01 mimicked the actions of ghrelin on Arc neurons in vitro and stimulated food intake and body weight gain in healthy rats. More importantly, HM01 attenuated cancer anorexia in a rat hepatoma model, and it positively affected metabolism, body weight development, and muscle wasting. Thus, ghrelin agonists, such as HM01, may be a useful therapeutic approach for the treatment of CACS and possibly other forms of pathological anorexia, malnutrition, and muscle wasting.

GRANTS

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DISCLOSURES

Claudio Pietra and Claudio Giuliano are employees of Helsinn Healthcare, Lugano, Switzerland.

AUTHOR CONTRIBUTIONS

T.B. and T.R. conception and design of research; T.B. and L.L. performed experiments; T.B. and L.L. analyzed data; T.B., L.L., and T.R. interpreted results of experiments; T.B. prepared figures; T.B. and L.L. drafted manuscript; T.B., C.P., C.G., T.A.L., and T.R. edited and revised manuscript; T.B., L.L., C.P., C.G., T.A.L., and T.R. approved final version of manuscript.

REFERENCES

1. **Andreyev HJ, Norman AR, Oates J, Cunningham D.** Why do patients with weight loss have a worse outcome when undergoing chemotherapy for gastrointestinal malignancies? *Eur J Cancer* 34: 503–509, 1998.
2. **Argiles JM, Busquets S, Stemmler B, Lopez-Soriano FJ.** Cancer cachexia: understanding the molecular basis. *Nat Rev Cancer* 14: 754–762, 2014.
3. **Borner T, Pinkernell S, Lutz TA, Riediger T.** Lipopolysaccharide inhibits ghrelin-excited neurons of the arcuate nucleus and reduces food intake via central nitric oxide signaling. *Brain Behav Immun* 26: 867–879, 2012.
4. **Chen JA, Splenser A, Guillory B, Luo J, Mendiratta M, Belinova B, Halder T, Zhang G, Li YP, Garcia JM.** Ghrelin prevents tumour- and cisplatin-induced muscle wasting: characterization of multiple mechanisms involved. *J Cachexia Sarcopenia Muscle* 6: 132–143, 2015.
5. **Cowley MA, Smith RG, Diano S, Tschop M, Pronchuk N, Grove KL, Strasburger CJ, Bidlingmaier M, Esterman M, Heiman ML, Garcia-Segura LM, Nillni EA, Mendez P, Low MJ, Sotonyi P, Friedman JM, Liu H, Pinto S, Colmers WF, Cone RD, Horvath TL.** The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. *Neuron* 37: 649–661, 2003.
6. **D'Onghia V, Leoncini R, Carli R, Santoro A, Giglioni S, Sorbellini F, Marzocca G, Bernini A, Campagna S, Marinello E, Vannoni D.** Circulating gastrin and ghrelin levels in patients with colorectal cancer: correlation with tumour stage, *Helicobacter pylori* infection and BMI. *Biomed Pharmacother* 61: 137–141, 2007.
7. **Davies JS, Kotokorpi P, Eccles SR, Barnes SK, Tokarczuk PF, Allen SK, Whitworth HS, Guschina IA, Evans BA, Mode A, Zigman JM, Wells T.** Ghrelin induces abdominal obesity via GHS-R-dependent lipid retention. *Mol Endocrinol* 23: 914–924, 2009.
8. **DeBoer MD, Zhu XX, Levasseur P, Meguid MM, Suzuki S, Inui A, Taylor JE, Halem HA, Dong JZ, Datta R, Culler MD, Marks DL.** Ghrelin treatment causes increased food intake and retention of lean body mass in a rat model of cancer cachexia. *Endocrinology* 148: 3004–3012, 2007.
9. **Dewys WD, Begg C, Lavin PT, Band PR, Bennett JM, Bertino JR, Cohen MH, Douglass HO Jr, Engstrom PF, Ezdinli EZ, Horton J, Johnson GJ, Moertel CG, Oken MM, Perlia C, Rosenbaum C, Silverstein MN, Skeel RT, Sponzo RW, Tormey DC.** Prognostic effect of weight loss prior to chemotherapy in cancer patients. Eastern Cooperative Oncology Group. *Am J Med* 69: 491–497, 1980.
10. **Filigheddu N, Gnocchi VF, Coscia M, Cappelli M, Porporato PE, Taulli R, Traini S, Baldanzi G, Chianale F, Cutrupi S, Arnoletti E, Ghe C, Fubini A, Surico N, Sinigaglia F, Ponzetto C, Muccioli G, Crepaldi T, Graziani A.** Ghrelin and des-acyl ghrelin promote differentiation and fusion of C2C12 skeletal muscle cells. *Mol Biol Cell* 18: 986–994, 2007.
11. **Garcia JM, Garcia-Touza M, Hijazi RA, Taffet G, Epner D, Mann D, Smith RG, Cunningham GR, Marcelli M.** Active ghrelin levels and active to total ghrelin ratio in cancer-induced cachexia. *J Clin Endocrinol Metab* 90: 2920–2926, 2005.

12. **Gordon JN, Green SR, Goggin PM.** Cancer cachexia. *Q J Med* 98: 779–788, 2005.
13. **Guan X, Yu H, Palyha O, McKee K, Feighner S, Sirinathsinghji D, Smith R, Van der Ploeg L, Howard A.** Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. *Brain Res Mol Brain Res* 48: 23–29, 1997.
14. **Hanada T, Toshinai K, Date Y, Kajimura N, Tsukada T, Hayashi Y, Kangawa K, Nakazato M.** Upregulation of ghrelin expression in cachectic nude mice bearing human melanoma cells. *Metabolism* 53: 84–88, 2004.
15. **Hanada T, Toshinai K, Kajimura N, Nara-Ashizawa N, Tsukada T, Hayashi Y, Osuye K, Kangawa K, Matsukura S, Nakazato M.** Anticachectic effect of ghrelin in nude mice bearing human melanoma cells. *Biochem Biophys Res Commun* 301: 275–279, 2003.
16. **Hewson AK, Dickson SL.** Systemic administration of ghrelin induces Fos and Egr-1 proteins in the hypothalamic arcuate nucleus of fasted and fed rats. *J Neuroendocrinol* 12: 1047–1049, 2000.
17. **Hewson AK, Viltart O, McKenzie DN, Dyball RE, Dickson SL.** GHRP-6-induced changes in electrical activity of single cells in the arcuate, ventromedial and periventricular nuclei neurones of a hypothalamic slice preparation in vitro. *J Neuroendocrinol* 11: 919–923, 1999.
18. **Huang Q, Fan YZ, Ge BJ, Zhu Q, Tu ZY.** Circulating ghrelin in patients with gastric or colorectal cancer. *Dig Dis Sci* 52: 803–809, 2007.
19. **Karasawa H, Pietra C, Giuliano C, Garcia-Rubio S, Xu X, Yakabi S, Tache Y, Wang L.** New ghrelin agonist, HM01 alleviates constipation and L-dopa-delayed gastric emptying in 6-hydroxydopamine rat model of Parkinson's disease. *Neurogastroenterol Motil* 26: 1771–1782, 2014.
20. **Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K.** Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 402: 656–660, 1999.
21. **Kumar NB, Kazi A, Smith T, Crocker T, Yu D, Reich RR, Reddy K, Hastings S, Exterman M, Balducci L, Dalton K, Bepler G.** Cancer cachexia: traditional therapies and novel molecular mechanism-based approaches to treatment. *Curr Treat Options Oncol* 11: 107–117, 2010.
22. **Lall S, Tung LY, Ohlsson C, Jansson JO, Dickson SL.** Growth hormone (GH)-independent stimulation of adiposity by GH secretagogues. *Biochem Biophys Res Commun* 280: 132–138, 2001.
23. **Laviano A, Meguid MM, Inui A, Muscaritoli M, Rossi-Fanelli F.** Therapy insight: Cancer anorexia-cachexia syndrome–when all you can eat is yourself. *Nat Clin Pract Oncol* 2: 158–165, 2005.
24. **Lugar CW, Clay MP, Lindstrom TD, Woodson AL, Smiley D, Heiman ML, Dodge JA.** Synthesis and biological evaluation of an orally active ghrelin agonist that stimulates food consumption and adiposity in rats. *Bioorg Med Chem Lett* 14: 5873–5876, 2004.
25. **Lundholm K, Gunnebo L, Korner U, Iresjo BM, Engstrom C, Hyltander A, Smedh U, Bosaeus I.** Effects by daily long term provision of ghrelin to unselected weight-losing cancer patients: a randomized double-blind study. *Cancer* 116: 2044–2052, 2010.
26. **Neary NM, Small CJ, Wren AM, Lee JL, Druce MR, Palmieri C, Frost GS, Ghatei MA, Coombes RC, Bloom SR.** Ghrelin increases energy intake in cancer patients with impaired appetite: acute, randomized, placebo-controlled trial. *J Clin Endocrinol Metab* 89: 2832–2836, 2004.
27. **Northrup R, Kuroda K, Duus EM, Barnes SR, Cheatham L, Wiley T, Pietra C.** Effect of ghrelin and anamorelin (ONO-7643), a selective ghrelin receptor agonist, on tumor growth in a lung cancer mouse xenograft model. *Support Care Cancer* 21: 2409–2415, 2013.
28. **Palus S, Schur R, Akashi YJ, Bockmeyer B, Datta R, Halem H, Dong J, Culler MD, Adams V, Anker SD, Springer J.** Ghrelin and its analogues, BIM-28131 and BIM-28125, improve body weight and regulate the expression of MuRF-1 and MAFbx in a rat heart failure model. *PloS One* 6: e26865, 2011.
29. **Palus S, von Haehling S, Doehner W, Datta R, Zhang J, Dong JZ, Culler MD, Anker SD, Springer J.** Effect of application route of the ghrelin analog BIM-28131 (RM-131) on body weight and body composition in a rat heart failure model. *Int J Cardiol* 168: 2369–2374, 2013.
30. **Perez-Tilve D, Heppner K, Kirchner H, Lockie SH, Woods SC, Smiley DL, Tschop M, Pfluger P.** Ghrelin-induced adiposity is independent of orexigenic effects. *FASEB J* 25: 2814–2822, 2011.
31. **Porporato PE, Filigheddu N, Reano S, Ferrara M, Angelino E, Gnocchi VF, Prodam F, Ronchi G, Fagoonee S, Fornaro M, Chianale F, Baldanzi G, Surico N, Sinigaglia F, Perroteau I, Smith RG, Sun Y, Geuna S, Graziani A.** Acylated and unacylated ghrelin impair skeletal muscle atrophy in mice. *J Clin Invest* 123: 611–622, 2013.
32. **Riediger T.** The receptive function of hypothalamic and brainstem centres to hormonal and nutrient signals affecting energy balance. *Proc Nutr Soc* 71: 463–477, 2012.
33. **Riediger T, Cordani C, Potes CS, Lutz TA.** Involvement of nitric oxide in lipopolysaccharide induced anorexia. *Pharmacol Biochem Behav* 97: 112–120, 2010.
34. **Riediger T, Traebert M, Schmid H, Scheel C, Lutz T, Scharrer E.** Site-specific effects of ghrelin on the neuronal activity in the hypothalamic arcuate nucleus. *Neurosci Lett* 341: 151–155, 2003.
35. **Ruud J, Blomqvist A.** Identification of rat brainstem neuronal structures activated during cancer-induced anorexia. *J Comp Neurol* 504: 275–286, 2007.
36. **Salome N, Hansson C, Taube M, Gustafsson-Ericson L, Egecioglu E, Karlsson-Lindahl L, Fehrentz JA, Martinez J, Perrissoud D, Dickson SL.** On the central mechanism underlying ghrelin's chronic pro-obesity effects in rats: new insights from studies exploiting a potent ghrelin receptor antagonist. *J Neuroendocrinol* 21: 777–785, 2009.
37. **Schiaffino S, Mammucari C.** Regulation of skeletal muscle growth by the IGF1-Akt/PKB pathway: insights from genetic models. *Skelet Muscle* 1: 4, 2011.
38. **Strassburg S, Anker SD, Castaneda TR, Burget L, Perez-Tilve D, Pfluger PT, Nogueiras R, Halem H, Dong JZ, Culler MD, Datta R, Tschop MH.** Long-term effects of ghrelin and ghrelin receptor agonists on energy balance in rats. *Am J Physiol Endocrinol Metab* 295: E78–E84, 2008.
39. **Strasser F, Lutz TA, Maeder MT, Thuerlimann B, Bueche D, Tschop M, Kaufmann K, Holst B, Brandle M, von Moos R, Demmer R, Cerny T.** Safety, tolerability and pharmacokinetics of intravenous ghrelin for cancer-related anorexia/cachexia: a randomised, placebo-controlled, double-blind, double-crossover study. *Br J Cancer* 98: 300–308, 2008.
40. **Sun Y, Wang P, Zheng H, Smith RG.** Ghrelin stimulation of growth hormone release and appetite is mediated through the growth hormone secretagogue receptor. *Proc Natl Acad Sci USA* 101: 4679–4684, 2004.
41. **Tisdale MJ.** Cachexia in cancer patients. *Nat Rev Cancer* 2: 862–871, 2002.
42. **Tschop M, Smiley D, Heiman M.** Ghrelin induces adiposity in rodents. *Nature* 407: 908–913, 2000.
43. **Tsubone T, Masaki T, Katsuragi I, Tanaka K, Kakuma T, Yoshimatsu H.** Ghrelin regulates adiposity in white adipose tissue and UCP1 mRNA expression in brown adipose tissue in mice. *Regul Pept* 130: 97–103, 2005.
44. **Vestergaard ET, Hansen TK, Gormsen LC, Jakobsen P, Moller N, Christiansen JS, Jorgensen JO.** Constant intravenous ghrelin infusion in healthy young men: clinical pharmacokinetics and metabolic effects. *Am J Physiol Endocrinol Metab* 292: E1829–E1836, 2007.
45. **Wang L, Saint-Pierre DH, Tache Y.** Peripheral ghrelin selectively increases Fos expression in neuropeptide Y-synthesizing neurons in mouse hypothalamic arcuate nucleus. *Neurosci Lett* 325: 47–51, 2002.
46. **Wang W, Andersson M, Iresjo BM, Lonnroth C, Lundholm K.** Effects of ghrelin on anorexia in tumor-bearing mice with eicosanoid-related cachexia. *Int J Oncol* 28: 1393–1400, 2006.
47. **Wisse BE, Frayo RS, Schwartz MW, Cummings DE.** Reversal of cancer anorexia by blockade of central melanocortin receptors in rats. *Endocrinology* 142: 3292–3301, 2001.
48. **Yasuda T, Masaki T, Kakuma T, Yoshimatsu H.** Centrally administered ghrelin suppresses sympathetic nerve activity in brown adipose tissue of rats. *Neurosci Lett* 349: 75–78, 2003.

8 Final Discussion and perspectives

8.1 Summary of the major findings

The current studies provided new insight into the pathophysiology of CACS and shed light on different strategies for its treatment. The first set of studies showed that the AP is involved in the central mediation of CACS in hepatoma TB rats. We also proposed MIC-1 as a potential tumor-released factor involved in the peripheral to central mediation of CACS in hepatoma TB rats. Moreover, we were able to provide first evidence of the central GLP-1 signaling within the AP/NTS region in the central mediation of CACS in this model. Furthermore, we provided a better characterization of the hepatoma tumor model by showing the presence of eating-independent effects on muscle and fat mass, as well as the development of tumor-induced food aversion. Finally, we highlighted the therapeutic efficacy of ghrelin-based approaches in the context of CACS by demonstrating the anti-CACS effects of the novel ghrelin analog HM01.

8.2 The relevance of brainstem and central GLP-1 signaling in CACS

8.2.1 The AP but not vagal afferents mediate CACS

The major goal of the first publication (i.e. chapter 5) presented in this thesis was to explore the relative contribution of AP and vagal afferents in the central mediation of CACS in hepatoma TB rats. By using advanced surgical approaches, we provided evidence that the AP is required for the central mediation of CACS in this model. Although changes in metabolic rate were not detected, the surgical lesion of the AP markedly attenuated cancer-dependent muscle degradation. This result is clinically relevant as loss of muscle mass is one of the most severe consequences of CACS.

Excessive muscle wasting compromises daily activities by increasing fatigue and weakness, which reduce quality of life [7]. In addition to its physical impact, muscle loss is also linked with an increase in chemotherapy-related side-effects and decreased survival rates [243]. The attenuation of anorexia induced by the lesion of the AP may contribute to the observed effects on muscles, suggesting that increased energy intake is able to partly counteract the tumor-induced muscle loss in this model.

In contrast, SDA did not affect CACS, indicating that the central mediation of CACS does not require vagal afferents signaling in this model. This is rather surprisingly given the importance of the vagus in the transmission of acute sickness, toxic and noxious signals to the brain. However, most of the studies investigating the involvement of the vagus nerve in this context were conducted in acute models of sickness, e.g., LPS, and cisplatin treatments or injection of pro-inflammatory cytokines [244-248].

Only two previous studies investigating the role of AP and vagal afferent signaling in the context of CACS were conducted in the past. However, the lesioning approach used in these studies (i.e. thermal lesion) was not specific for the AP because the lesion included major parts of the NTS [249]. Furthermore, the experimental procedures used to block vagal afferent signaling (i.e. perivagal capsaicin injection and total subdiaphragmatic vagotomy) were also not specific and selective [250]. In addition, the results of these studies are probably the consequences of increased estrogen levels rather than specific tumor-dependent signaling mechanisms.

In our studies, the AP was removed via vacuum aspiration, allowing the specific removal of the AP without damaging the NTS and thus compromising its structural integrity [114]. In addition, to surgically disrupt vagal afferent signaling, we used the SDA technique. SDA is the most specific surgical deafferentation method available and consists in a unilateral intracranial transection of the vagal afferent rootlets and an ipsilateral transection of the dorsal subdiaphragmatic trunk of the vagus nerve. Hence, this procedure disrupts all subdiaphragmatic vagal afferents while leaving half of vagal efferents intact, minimizing the impairment of gastrointestinal motor function that usually occurs following complete vagotomies [251]. Altogether, the combination of both approaches allowed us to dissociate the contribution of the AP from vagal afferents in a model that mimics better the situation in human cancer patients.

In conclusion, our findings demonstrate the importance of the AP in the mediation of cancer-dependent anorexia and body weight loss. The underlying local neuronal

signaling within the AP/NTS region (e.g. GLP-1) represents promising therapeutic targets for the treatment of CACS.

8.2.2 GLP-1 expressing neurons in NTS mediate CACS

The overall aim of the second set of studies (i.e. chapter 6) was to identify the putative neuronal mechanism responsible for the previously observed AP-dependent effects in this model for CACS. These studies provided the first evidence that the central GLP-1 system is required for cancer anorexia and body weight loss in hepatoma TB rats. Specifically, a pharmacological blockade of the brainstem GLP-1R signaling, as well as a genetic inhibition of the NTS GLP-1 production, attenuated anorexia, body weight loss, muscle mass loss and fat mass wasting induced by tumor growth. Both approaches did not affect food intake and body weight under non-pathological conditions, underlying the specific role of GLP-1 in the etiology of CACS in this model. These results complement our findings of the involvement of the AP in CACS and also support the role of GLP-1 signaling within the AP/NTS for the mediation of CACS in this model. In line with the importance of GLP-1 signaling in the mediation of aversion, emesis and nausea, we also provided evidence demonstrating the presence of food aversion during CACS in hepatoma TB rats.

The outcomes of the pharmacological and genetic blockade of brainstem GLP-1 signaling were similar. In both studies, tumor-induced anorexia was markedly attenuated. Moreover, either a positive treatment effect on lean mass or muscle mass in addition to a preservation of fat mass was observed. Ex-9 did not alter metabolic rate in TB rats. The anti-CACS effect of GLP-1 blockade is primarily based on higher food intake, which consequently leads to a preservation of fat mass and lean mass. Collectively, the use of pharmacological and genetic approaches strengthened the relevance of GLP-1 signaling in the etiology of CACS. Furthermore, the outcome of the PPG knockdown (PPG-KD) study confirmed the contribution of NTS-derived GLP-1 in the central mediation of CACS, further supporting the crucial role of brainstem structures in the pathophysiology of CACS.

At least in lean ad libitum fed rats, the blockade of brainstem GLP-1R did not affect energy intake or body weight development [144, 224, 225]. Our findings are consistent with these studies since neither Ex-9 treatment nor PPG-KD affected food intake or body weight in healthy rats. Based on these observations, brainstem GLP-1 does not seem to be required for the maintenance of energy balance in healthy animals maintained under normal conditions. Hence, the anti-CACS effects described here are due to a specific blockade of a pathological activation of the brainstem GLP-1 system, and not secondary to alterations in eating behavior unrelated to CACS per se. The central GLP-1 inhibition does not compromise the normal control of eating but selectively blocks the tumor-dependent anorectic mechanisms that override physiological signaling.

8.2.3 Central GLP-1 signaling in cancer-related malaise

Previous studies conducted in rodents demonstrated the development of CTA as a consequence of cancer [236, 252]. Our results are in line with these previous reports. TB rats avoided the flavour that was preferred before tumour growth and presented during the conditioning phase, clearly showing the development of CTA. Evidence suggests that the central GLP-1 system is implicated in malaise, nausea and in the mediation of aversion, which contribute to the reduction of appetite and energy intake induced by different toxic agents [141, 142, 144]. Whether the suppression of food intake that occurred in TB animals is due to CTA is difficult to examine under our experimental conditions. The severity of the anorectic response was not decreased when the diet was changed or an additional diet was offered, indicating that changes in the diet's flavour did not attenuate anorexia. Nevertheless, our results point to a GLP-1 dependent mediation of CTA, which could be examined by combining GLP-1 blockade with a CTA paradigm.

Food aversion following malaise is a known phenomenon, which not only occurs under experimental conditions but also in cancer patients and may act together with anorexia to suppress appetite and energy intake [106, 234]. The development of CTA, nausea and emesis can be a consequence of the cancer itself but also secondary to anti-cancer treatments [234-239]. For instance, chemotherapeutic agents are often accompanied by

these severe side effects, which reduce quality of life and may lead to treatment delays or even treatment abortion [253]. Chemotherapy-induced nausea, food aversion and vomiting are ranked as the biggest patients concerns in anticipation of cancer treatment [254]. More than 90% of patients treated with cisplatin suffer from chemotherapy-induced nausea, food aversion and vomiting, and approximately 50% develop profound anorexia, which still persists despite anti-emetic pharmacological treatments [255, 256]. Indeed, both serotonin and neurokinin-1 receptor antagonists, two classes of antiemetic medications, reduce acute chemotherapy-induced emesis and nausea but fail to effectively suppress the delayed malaise phase that persist up to 7 days after chemotherapy [257-260].

The central GLP-1 system is activated by cisplatin, and brainstem GLP-1R signaling partially mediates the delayed malaise phase [142]. Our findings complement the outcomes of this study and highlight the possibility that brainstem GLP-1 might be common mediator of anorexia and malaise in different chronic disease models, including chemotherapy-induced malaise and CACS.

8.2.4 Translation of GLP-1-based approaches to human cancer patients

The surgical removal of the AP in human cancer patients is not an option. Although AP lesions in humans were performed in the past to treat continuous nausea and vomiting, the side effects were severe [96]. A pharmacological inhibition of the neuronal population that is activated during cancer (i.e. GLP-1R-expressing neurons) obviously is a better approach. Although we did not perform peripheral Ex-9 treatments in our current studies, GLP-1 and its agonists can target GLP-1R located in the AP after peripheral administration [94, 125, 261, 262]. Therefore, one can assume that Ex-9 would exert its central effects after peripheral administration as well. Furthermore, systemic administration of Ex-9 could have additional peripheral effects that were not engaged after central delivery. Increased expression of uncoupling proteins (UCPs) in white and brown adipose tissues as well in muscles has been suggested as potential contributing factors to CACS [263-265]. Although the physiological and pathological relevance of UCPs in muscles still requires deeper investigation, peripheral treatment with Ex-4 increased energy expenditure by upregulating UCP-1 expression in muscle

likely via a direct mechanism [233]. Therefore, inhibition of peripheral GLP-1 signaling could potentially counteract muscle mass loss by decreasing energy expenditure in CACS patients.

Importantly, GLP-1-based approaches could be more specific and may have less side-effects compared to the current therapeutic options. For instance, megestrol acetate treatment, the most used drug for the treatment of CACS, is linked with serious side-effects that may compromise life quality, such as nausea, vomiting and confusion, but also many severe life threatening side effects including pulmonary edema, thrombosis and heart failure [17]. In contrast, the only potential side effect noticed during Ex-9 treatment in healthy and obese humans so far was a mild hyperglycemic state, which it can be easily be prevented by insulin co-administration [266-268]. While more pre-clinical and clinical studies in the context of cancer are required to shed more light on the beneficial effects of Ex-9 peripheral treatment as well as its safety and its efficacy, our findings suggest that GLP-1R antagonists may be therapeutically useful for the treatment of CACS in humans.

8.2.5 Downstream projection sites of tumor-activated AP/NTS neurons as potential therapeutic targets for the treatment of CACS

The PBN is an important downstream target of signals originated in the AP/NTS region following different anorectic stimuli (e.g. amylin, CCK, LiCl, LPS) and its role in the regulation of eating and in the mediation of anorexia and CTA is well established [269-272]. c-Fos expression in the PBN was observed in hepatoma TB rats. In particular, c-Fos labeling was located in the external lateral nucleus (i.e. LPBN), corresponding to the main ascending terminal target of the medial part of the NTS and the AP [111]. This strongly suggests that activated AP/NTS neurons are directly involved in the activation of LPBN neurons. Thus, the LPBN is a potential site where neuronal signals may converge and trigger reduced eating.

A population of LPBN neurons expresses the calcitonin related peptide (CGRP) and sends projections to different brain areas including the central amygdala. Central injection of CGRP in the amygdala reduced food intake, while inhibition of LPBN CGRP

neurons blunted anorexia induced by the satiation hormone CCK as well other anorectic substances such as LiCl [273]. Conversely, activation of CGRP LPBN neurons decreases feeding [271]. Surgical, pharmacological and genetic apporaches such as LPBN lesion, selective LPBN CGRP knockdown and central CGRP receptor antagonist treatment can be use to investigate the involvement of LPBN CGRP signaling in hepatoma TB rats and possibly link the signals originating in the AP/NTS with the CGRP signaling in the LPBN. If this hypothesis is correct, pharmacological approaches targeting CGRP signaling might represent another potential therapeutic option for the treatment of CACS.

8.2.6 MIC-1 as the putative humoral factor mediating CACS

The inflammatory peripheral mediator responsible for the neuropathological changes following tumor growth was unknown in this model, as no "classical" inflammatory cytokine was increased in the circulation [111]. We identified MIC-1 as a potential mediator of CACS in hepatoma TB rats by showing that MIC-1 levels were elevated during cancer. Moreover, its levels correlated with the severity of the anorectic response, tumor progression and tumor weight. Further studies are still required to demonstrate a direct involvement of MIC-1 in the pathophysiology of CACS in this model. Unfortunately, MIC-1 binds to an orphan receptor. Hence its receptor cannot be targeted by antagonists to confirm that endogenous MIC-1 signaling is required for the central mediation of CACS in our model [39]. At present, gene-targeting approaches appear to be the only practicable method to chronically disrupt MIC-1 signaling in rodents. While such studies have not yet been conducted, our results are consistent with previous findings showing that the anorectic effect of exogenous MIC-1 required an intact AP/NTS [101].

8.2.7 Proposed mechanism for CACS in hepatoma TB rats

The results obtained following genetic or pharmacological inhibition of central GLP-1 signaling are similar to those of our AP-lesion study in respect to many of the measured parameters. Consequently, the comparable outcomes of these studies are consistent

with the hypothesis that CACS in this model is mediated by GLP-1-ergic projections from the NTS to the AP. One could speculate that other brain areas that are targeted by NTS GLP-ergic projections might also contribute to the mediation of CACS. However, due to the rostral-to-caudal flow of the cerebrospinal fluid, Ex-9 infused into the fourth ventricle is unlikely to efficiently block GLP-1R signaling in forebrain structures.

Although GLP-1 signaling in the AP/NTS region was identified as crucial for the central mediation of CACS in this model, the underlying mechanism that stimulates NTS GLP-1 neurons and that, leads to the activation of the AP/NTS region, still remains unknown. As mentioned in the introduction, growing evidence suggests a role of MIC-1 in the central mediation of CACS via AP/NTS signaling. Direct conclusive evidence is still lacking. MIC-1 responsive neurons in the NTS are mostly non-catecholaminergic [101]. Interestingly, NTS GLP-1 neurons do not co-express catecholaminergic neurotransmitters [222]. These observations suggest a possible functional link between MIC-1, AP/NTS activation and brainstem GLP-1-mediated mediation of CACS. The dendrites of NTS GLP-1 neurons extend into the AP [131]. Thus, MIC-1 could directly activate GLP-1 expressing neurons in the NTS. This may then lead to the activation of GLP-1R expressing neurons in the AP and in its downstream projection sites (e.g. LPBN) that are involved in the control of food intake (Fig. 1).

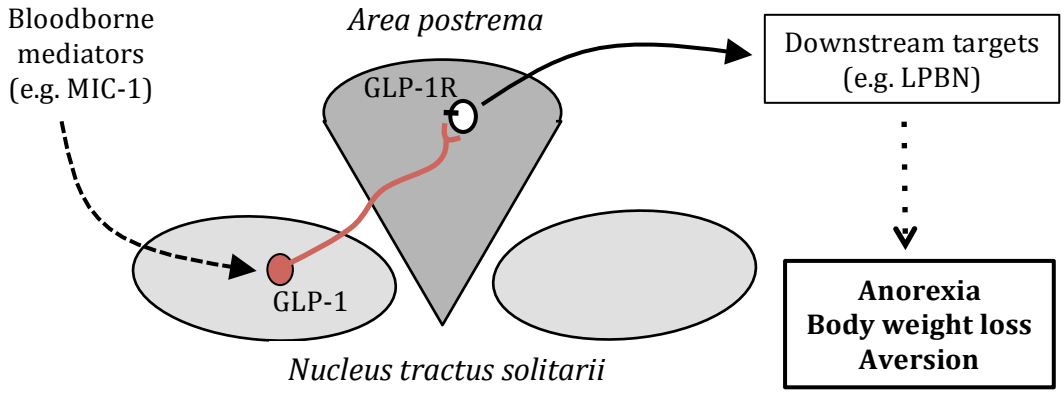

***Fig. 1*. Proposed mechanism for CACS in hepatoma TB rats:** *MIC-1 directly activates GLP-1 expressing neurons located in the NTS. This leads to GLP-1 release in the AP and a subsequent activation of GLP-1R neurons. The signal is then transmitted to downstream target areas involved in the control of food intake, contributing to anorexia, CTA, and eventually body weight loss.*

8.3 The evaluation of the ghrelin analog HM01 for the treatment of CACS

Ghrelin-based approaches represent a promising strategy for the treatment of CACS. The positive effects of ghrelin treatments have been demonstrated in different models for CACS, as well as in human cancer patients (see table 1). Unfortunately, however, the clinical use of ghrelin is limited due to its short halftime and its peptidergic nature that excludes its oral delivery.

Our manuscript (i.e. chapter 7) provided the first characterization of the synthetic GHSR agonist HM01 in the context of CACS. Our major aim was to evaluate the ability of the novel ghrelin agonist HM01 to counteract tumor-induced anorexia and body weight loss. We demonstrated that HM01 increased food intake and body weight in healthy rats. Moreover, HM01 also improved both total fat and lean volumes and gastrocnemius mass. More importantly, HM01 was proven effective in preventing tumor-induced body weight loss and muscle degradation by attenuating anorexia and by reducing energy expenditure.

8.3.1 HM01 attenuated tumor-induced anorexia and reduced energy expenditure

There is some evidence for a decreased responsiveness to ghrelin treatment under tumor conditions. In fact, systemic and central ghrelin administration in different rodent models for CACS failed to stimulate eating if compared to healthy controls [166, 274]. This lack of ghrelin responsiveness was also observed in cancer patients [275]. Furthermore, in some models for CACS, which are characterized by higher levels of ghrelin, increased endogenous serum ghrelin levels accompanied this reduced effectiveness [166, 274, 276]. These findings might indicate a state of ghrelin resistance or at least reduced ghrelin responsiveness under tumor conditions. However, in contrast to what has been observed in these studies, the relative increase in eating observed in HM01-treated TB rats was comparable to the increase observed in healthy animals. Further, under our testing conditions, the effects of HM01 on eating and body

weight persisted until the end of the experiment. For these reasons, or results do not suggest the presence of a profound ghrelin resistance.

Interestingly, HM01-treatment reduced energy expenditure. While the ghrelin effects on energy expenditure are known, our study is the first showing a beneficial effect of a ghrelin-based therapy on energy expenditure in the context of CACS. Therefore, the observed preservation of body weight following tumor-induction could be partly the consequence of both higher food intake and reduced energy metabolism.

8.3.2 HM01 prevents muscle mass degradation

HM01 not only stimulated muscle growth in healthy animals but it also increased muscle mass in TB rats. As mentioned in the introduction, ghrelin exerts anabolic actions via GH/IGF-1 signaling. Since HM01 is a potent stimulator of GH release (Helsinn Healthcare SA, unpublished findings), the beneficial effect of HM01 on muscle mass is likely to be at least be partly mediated by increased GH/IGF-1 signaling, although future studies are required to confirm this.

In addition to its orexigenic and anabolic actions, ghrelin-based approaches might have additional anti-CACS properties due to ghrelin's anti-inflammatory properties [277]. Hence, HM01-induced inhibition of central and peripheral inflammatory signaling cascades might not only indirectly blunt muscle degradation by attenuating anorexia, but also prevent direct cytokine-mediated muscle wasting. The only circulating cytokine that was found evaluated in this model was MIC-1. No increase in systemic levels of IL-6, TNF-α, IL-1β and IFN-γ, IL-6 has been reported [111]. Therefore, it is unlikely that the effects observed in our study are due to HM01 anti-inflammatory actions. Other models, characterized by systemic cytokine release, are more appropriate for testing the anti-inflammatory properties of HM01. Similar studies using the mouse C26 model, which is characterized by systemic cytokine release, showed a reduction of TNF-α, and IL-1β levels after ghrelin administration [79]. HM01 treatment in those animals increased food intake, body weight, fat and muscle mass while it decreased energy expenditure. These effects appeared to be independent of the potential anti-inflammatory effects of HM01 as IL-6, MIC-1, MuRF-1 or MAFbx levels were not affected by the treatment [278].

8.3.3 The efficacy of ghrelin-based therapies was recently confirmed in phase III studies

The use of ghrelin-based therapies to effectively prevent and reverse CACS in humans was substantiated by two randomized, double-blind phase III clinical trials (i.e. ROMANA 1 and ROMANA 2) [205]. In these studies, which are so far the largest and the longest trial conducted in the context of CACS, the novel, orally available, peptide-derived ghrelin mimetic anamorelin was tested. Anamorelin was administered daily for 12 weeks in patients with inoperable non-small-cell lung cancer and CACS. Patients receiving anamorelin increased body weight and lean mass but also had an improvement in their anorexia symptoms (e.g. appetite, nausea and food aversion), which were evaluated using the Functional Assessment of Cancer Therapy (FACT) questionnaire [279]. Unfortunately, anamorelin failed to show significant improvements in muscle strength and fatigue, probably as a consequence of the high degree of subject variability and cancer treatments.

Although HM01 was not tested in humans yet, pre-clinical studies anticipate a higher brain permeability and bioavailability of HM01 compared to anamorelin (Helsinn Healthcare SA, unpublished results). Hence, one could assume that HM01 would have a greater impact on increasing eating and GH-release and on decreasing energy expenditure. If this were confirmed in humans, HM01 might represent a fundamental step toward a more efficient clinical management of CACS.

8.3.4 Ghrelin and ghrelin mimetics are safe and well tolerated in humans

One of the major concerns regarding ghrelin-based approaches is the possibility to promote tumor growth via GH/IGF-1 signaling. Nevertheless, all experiments conducted in different rodent models have so far demonstrated the safety of ghrelin-based therapeutic approaches without evidence of increased tumor progression following 1-14 days of treatment [280-283]. The lack of differences in tumor weight in our study is in line with these findings. Even more significant are recent clinical data revealing that the overall survival, tumor size or tumor progression markers were not increased after sustained and prolonged treatment with ghrelin or ghrelin mimetics [284, 285].

Additionally, in all clinical studies, no severe side effects were induced by ghrelin-based treatments [205, 275, 284-287]. In healthy subjects side effects were extremely rare, with most common adverse events being mild headache and stomachache, which both regressed spontaneously [288-291]. A recent meta-analysis of all published anamorelin phase II and phase III randomized trials demonstrated that anamorelin induced fewer adverse events compared to placebo [292]. This outcome confirms the safety and tolerability of anamorelin for long-term applications in humans. One can speculate that other GHSR analogs, including HM01, would be safe and tolerated in cancer patients as well.

8.4 Significance of these studies for other form of anorexia-cachexia

While this thesis focused specifically on CACS, it needs to be mentioned that anorexia-cachexia syndrome (ACS) is also associated with other chronic diseases such as bacterial and parasitic infection, acquired immune deficiency syndrome (AIDS), chronic obstructive pulmonary disease (COPD), chronic inflammatory bowel disease, chronic liver disease, chronic cardiovascular disease, rheumatoid arthritis and many others. Between 10% and 40% of patients affected by these disorders experience malnutrition and loss of body weight requiring medical treatment. Altogether, in the US alone, more than 30 million of people suffer from ACS [293]. Moreover, anorexia, nausea and vomiting are caused by a wide variety of pharmacotherapies (e.g. lithium-based psychoactive agents and chemotherapeutic drugs). The GLP-1 antagonism and ghrelin-based treatment approaches examined here focused on anorexia, malaise and body weight loss in the context of cancer. However, the neuronal pathways investigated may have broader implications for all these diseases and treatments that cause these side effects.

8.5 Overall conclusions

CACS severely deteriorates the clinical status and promotes morbidity and mortality with a very high prevalence in particular types of cancers [1, 4]. Importantly, CACS has been reported by cancer patients to be the major detrimental factor affecting quality of life [7]. Unfortunately, the clinical management of CACS is still inadequate and currently available therapies are insufficient. The most widely used drugs such as progesterone and corticoid-based agents cause only a transient stimulation of food intake and a moderate increase body weight and failed to show an improvement in the general quality of life. Unfortunately, other treatment approaches have not yet been developed into effective therapies [8]. A better understanding of the pathophysiology of CACS is a crucial step towards the development of effective and specific therapeutic approaches.

In this dissertation, we provided new insight on the pathophysiology of CACS and proposed different strategies for its treatment. We substantiated the fundamental role of the AP in the mediation of CACS and we provided evidence for a role of central GLP-1 signaling in hepatoma TB rats. Furthermore, we demonstrated the therapeutic efficacy of the novel ghrelin analog HM01 in the context of CACS. Both ghrelin and GLP-1-based approaches represent promising options for the treatment of CACS and possibly other forms of disease-related anorexia.

9 List of abbreviations

AgRP	Neuropeptides agouti-related peptide
AP	Area postrema
APX	Area postrema lesion
Arc	Arcuate nucleus
BAT	Brown adipose tissue
BBB	Blood-brain barrier
CACS	Cancer anorexia-cachexia syndrome
CART	cocaine- and amphetamine-regulated transcript
CCK	Cholecystokinin
CGRP	Calcitonin related peptide
CNS	Central nervous system
CTA	Conditioned taste aversion
DMN	Dorsal motor nucleus of the vagus
Ex-4	Exendin-4
Ex-9	Exendin-9
GDF15	Growth differentiation factor 15
GH	Growth hormone
GHSR	Growth hormone secretagouge receptor
GLP-1	Glucagon-like peptide-1
GLP-1R	Glucagon-like peptide-1 receptor
IFN-γ	Interferon γ
IGF-1	Insulin-like growth factor-1
IL-1β	Interleukin 1β
IL-6	Interleukin 6
LiCl	Lithium chloride
LPBN	Lateral parabrachial nucleus
LPS	Lipopolysaccharide
MAFbx/atrogin-1	Muscle atrophy F-box protein
MC4R	Melanocortin 4 receptor
MIC-1	Macrophage inhibitory cytokine-1

MURF-1	Muscle RING finger protein-1
NO	Nitric oxide
NPY	Neuropeptide Y
NTB	Non-tumor-bearing
NTS	Nucleus tractus solitarii
POMC	Pro-opiomelanocortin
PPG	Pre-pro-glucagon
PVN	Paraventricular nucleus
RER	Respiratory exchange ration
sCVO	Sensory circumventricular organs
SDA	Subdiaphragmatic vagal deafferentation
STAT	Signal transducers and activators of transcription
TB	Tumor-bearing
TNF-α	Tumor necrosis factor α
UCP-1	Uncoupling protein-1
αMSH	α melanocyte stimulating hormone

10 References

1. Laviano A, Meguid MM, Rossi-Fanelli F. Cancer anorexia: clinical implications, pathogenesis, and therapeutic strategies. Lancet Oncol. 2003;4(11):686-94.
2. Fearon K, Strasser F, Anker SD, Bosaeus I, Bruera E, Fainsinger RL, Jatoi A, Loprinzi C, MacDonald N, Mantovani G, Davis M, Muscaritoli M, Ottery F, Radbruch L, Ravasco P, Walsh D, Wilcock A, Kaasa S, Baracos VE. Definition and classification of cancer cachexia: an international consensus. Lancet Oncol. 2011;12(5):489-95.
3. von Haehling S, Anker MS, Anker SD. Prevalence and clinical impact of cachexia in chronic illness in Europe, USA, and Japan: facts and numbers update 2016. Journal of cachexia, sarcopenia and muscle. 2016;7(5):507-9.
4. Sun L, Quan XQ, Yu S. An Epidemiological Survey of Cachexia in Advanced Cancer Patients and Analysis on Its Diagnostic and Treatment Status. Nutrition and cancer. 2015;67(7):1056-62.
5. Dewys WD, Begg C, Lavin PT, Band PR, Bennett JM, Bertino JR, Cohen MH, Douglass HO, Jr., Engstrom PF, Ezdinli EZ, Horton J, Johnson GJ, Moertel CG, Oken MM, Perlia C, Rosenbaum C, Silverstein MN, Skeel RT, Sponzo RW, Tormey DC. Prognostic effect of weight loss prior to chemotherapy in cancer patients. Eastern Cooperative Oncology Group. The American journal of medicine. 1980;69(4):491-7.
6. Argiles JM, Busquets S, Stemmler B, Lopez-Soriano FJ. Cancer cachexia: understanding the molecular basis. Nature reviews Cancer. 2014;14(11):754-62.
7. Tan BH, Fearon KC. Cachexia: prevalence and impact in medicine. Curr Opin Clin Nutr Metab Care. 2008;11(4):400-7.
8. Fearon K, Arends J, Baracos V. Understanding the mechanisms and treatment options in cancer cachexia. Nature reviews Clinical oncology. 2013;10(2):90-9.
9. Andreyev HJ, Norman AR, Oates J, Cunningham D. Why do patients with weight loss have a worse outcome when undergoing chemotherapy for gastrointestinal malignancies? European journal of cancer. 1998;34(4):503-9.
10. Ross PJ, Ashley S, Norton A, Priest K, Waters JS, Eisen T, Smith IE, O'Brien ME. Do patients with weight loss have a worse outcome when undergoing chemotherapy for lung cancers? British journal of cancer. 2004;90(10):1905-11.
11. Tisdale MJ. Cachexia in cancer patients. Nature reviews Cancer. 2002;2(11):862-71.
12. Dodson S, Baracos VE, Jatoi A, Evans WJ, Cella D, Dalton JT, Steiner MS. Muscle wasting in cancer cachexia: clinical implications, diagnosis, and emerging treatment strategies. Annual review of medicine. 2011;62:265-79.
13. Hinsley R, Hughes R. 'The reflections you get': an exploration of body image and cachexia. International journal of palliative nursing. 2007;13(2):84-9.
14. Fouladiun M, Korner U, Gunnebo L, Sixt-Ammilon P, Bosaeus I, Lundholm K. Daily physical-rest activities in relation to nutritional state, metabolism, and quality of life in cancer patients with progressive cachexia. Clinical cancer research : an official journal of the American Association for Cancer Research. 2007;13(21):6379-85.
15. Vaughan VC, Martin P, Lewandowski PA. Cancer cachexia: impact, mechanisms and emerging treatments. Journal of cachexia, sarcopenia and muscle. 2013;4(2):95-109.
16. Kotler DP. Cachexia. Annals of internal medicine. 2000;133(8):622-34.
17. Ruiz Garcia V, Lopez-Briz E, Carbonell Sanchis R, Gonzalvez Perales JL, Bort-Marti S. Megestrol acetate for treatment of anorexia-cachexia syndrome. The Cochrane database of systematic reviews. 2013(3):CD004310.

18. Loprinzi CL, Kugler JW, Sloan JA, Mailliard JA, Krook JE, Wilwerding MB, Rowland KM, Jr., Camoriano JK, Novotny PJ, Christensen BJ. Randomized comparison of megestrol acetate versus dexamethasone versus fluoxymesterone for the treatment of cancer anorexia/cachexia. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 1999;17(10):3299-306.
19. Cannabis In Cachexia Study G, Strasser F, Luftner D, Possinger K, Ernst G, Ruhstaller T, Meissner W, Ko YD, Schnelle M, Reif M, Cerny T. Comparison of orally administered cannabis extract and delta-9-tetrahydrocannabinol in treating patients with cancer-related anorexia-cachexia syndrome: a multicenter, phase III, randomized, double-blind, placebo-controlled clinical trial from the Cannabis-In-Cachexia-Study-Group. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2006;24(21):3394-400.
20. Eleutherakis-Papaiakovou V, Bamias A, Dimopoulos MA. Thalidomide in cancer medicine. Annals of oncology : official journal of the European Society for Medical Oncology. 2004;15(8):1151-60.
21. Laviano A, Meguid MM, Inui A, Muscaritoli M, Rossi-Fanelli F. Therapy insight: Cancer anorexia-cachexia syndrome--when all you can eat is yourself. Nature clinical practice Oncology. 2005;2(3):158-65.
22. Schwartz MW, Woods SC, Porte D, Jr., Seeley RJ, Baskin DG. Central nervous system control of food intake. Nature. 2000;404(6778):661-71.
23. Sandoval D, Cota D, Seeley RJ. The integrative role of CNS fuel-sensing mechanisms in energy balance and glucose regulation. Annual review of physiology. 2008;70:513-35.
24. Cummings DE, Overduin J. Gastrointestinal regulation of food intake. J Clin Invest. 2007;117(1):13-23.
25. Woods SC, Lutz TA, Geary N, Langhans W. Pancreatic signals controlling food intake; insulin, glucagon and amylin. Philosophical transactions of the Royal Society of London Series B, Biological sciences. 2006;361(1471):1219-35.
26. Friedman JM, Halaas JL. Leptin and the regulation of body weight in mammals. Nature. 1998;395(6704):763-70.
27. Grill HJ, Hayes MR. The nucleus tractus solitarius: a portal for visceral afferent signal processing, energy status assessment and integration of their combined effects on food intake. Int J Obes (Lond). 2009;33 Suppl 1:S11-5.
28. Cowley MA, Smart JL, Rubinstein M, Cerdan MG, Diano S, Horvath TL, Cone RD, Low MJ. Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. Nature. 2001;411(6836):480-4.
29. Varela L, Horvath TL. Leptin and insulin pathways in POMC and AgRP neurons that modulate energy balance and glucose homeostasis. EMBO reports. 2012;13(12):1079-86.
30. Stephens TW, Basinski M, Bristow PK, Bue-Valleskey JM, Burgett SG, Craft L, Hale J, Hoffmann J, Hsiung HM, Kriauciunas A, et al. The role of neuropeptide Y in the antiobesity action of the obese gene product. Nature. 1995;377(6549):530-2.
31. Mizuno TM, Mobbs CV. Hypothalamic agouti-related protein messenger ribonucleic acid is inhibited by leptin and stimulated by fasting. Endocrinology. 1999;140(2):814-7.
32. Muller TD, Nogueiras R, Andermann ML, Andrews ZB, Anker SD, Argente J, Batterham RL, Benoit SC, Bowers CY, Broglio F, Casanueva FF, D'Alessio D, Depoortere I, Geliebter A, Ghigo E, Cole PA, Cowley M, Cummings DE, Dagher A, Diano S, Dickson SL, Dieguez C, Granata R, Grill HJ, Grove K, Habegger KM, Heppner K, Heiman ML, Holsen L, Holst B, Inui A, Jansson JO, Kirchner H, Korbonits M, Laferrere B, LeRoux CW, Lopez M, Morin S, Nakazato M, Nass R, Perez-Tilve D, Pfluger PT, Schwartz TW, Seeley RJ,

Sleeman M, Sun Y, Sussel L, Tong J, Thorner MO, van der Lely AJ, van der Ploeg LH, Zigman JM, Kojima M, Kangawa K, Smith RG, Horvath T, Tschop MH. Ghrelin. Molecular metabolism. 2015;4(6):437-60.
33. Traebert M, Riediger T, Whitebread S, Scharrer E, Schmid HA. Ghrelin acts on leptin-responsive neurones in the rat arcuate nucleus. J Neuroendocrinol. 2002;14(7):580-6.
34. Tisdale MJ. Cachexia in cancer patients. Nat Rev Cancer. 2002;2(11):862-71.
35. Inui A. Cancer anorexia-cachexia syndrome: are neuropeptides the key? Cancer research. 1999;59(18):4493-501.
36. Ramos EJ, Suzuki S, Marks D, Inui A, Asakawa A, Meguid MM. Cancer anorexia-cachexia syndrome: cytokines and neuropeptides. Curr Opin Clin Nutr Metab Care. 2004;7(4):427-34.
37. Langhans W. Anorexia of infection: current prospects. Nutrition. 2000;16(10):996-1005.
38. Plata-Salaman CR. Anorexia during acute and chronic disease. Nutrition. 1996;12(2):69-78.
39. Breit SN, Johnen H, Cook AD, Tsai VW, Mohammad MG, Kuffner T, Zhang HP, Marquis CP, Jiang L, Lockwood G, Lee-Ng M, Husaini Y, Wu L, Hamilton JA, Brown DA. The TGF-beta superfamily cytokine, MIC-1/GDF15: a pleotrophic cytokine with roles in inflammation, cancer and metabolism. Growth factors. 2011;29(5):187-95.
40. Plata-Salaman CR, Ffrench-Mullen JM. Intracerebroventricular administration of a specific IL-1 receptor antagonist blocks food and water intake suppression induced by interleukin-1 beta. Physiol Behav. 1992;51(6):1277-9.
41. Yang ZJ, Blaha V, Meguid MM, Laviano A, Oler A, Zadak Z. Interleukin-1alpha injection into ventromedial hypothalamic nucleus of normal rats depresses food intake and increases release of dopamine and serotonin. Pharmacology, biochemistry, and behavior. 1999;62(1):61-5.
42. Sonti G, Ilyin SE, Plata-Salaman CR. Anorexia induced by cytokine interactions at pathophysiological concentrations. Am J Physiol. 1996;270(6 Pt 2):R1394-402.
43. Watkins LR, Maier SF, Goehler LE. Cytokine-to-brain communication: a review & analysis of alternative mechanisms. Life Sci. 1995;57(11):1011-26.
44. Hopkins SJ, Rothwell NJ. Cytokines and the nervous system. I: Expression and recognition. Trends Neurosci. 1995;18(2):83-8.
45. Brady LS, Lynn AB, Herkenham M, Gottesfeld Z. Systemic interleukin-1 induces early and late patterns of c-fos mRNA expression in brain. J Neurosci. 1994;14(8):4951-64.
46. Niimi M, Wada Y, Sato M, Takahara J, Kawanishi K. Effect of continuous intravenous injection of interleukin-6 and pretreatment with cyclooxygenase inhibitor on brain c-fos expression in the rat. Neuroendocrinology. 1997;66(1):47-53.
47. Emch GS, Hermann GE, Rogers RC. TNF-alpha-induced c-Fos generation in the nucleus of the solitary tract is blocked by NBQX and MK-801. Am J Physiol Regul Integr Comp Physiol. 2001;281(5):R1394-400.
48. Katafuchi T, Motomura K, Baba S, Ota K, Hori T. Differential effects of tumor necrosis factor-alpha and -beta on rat ventromedial hypothalamic neurons in vitro. Am J Physiol. 1997;272(6 Pt 2):R1966-71.
49. Plata-Salaman CR, ffrench-Mullen JM. Interleukin-1 beta inhibits Ca2+ channel currents in hippocampal neurons through protein kinase C. Eur J Pharmacol. 1994;266(1):1-10.
50. Plata-Salaman CR. Cytokine-induced anorexia. Behavioral, cellular, and molecular mechanisms. Ann N Y Acad Sci. 1998;856:160-70.

51. McCarthy DO. Cytokines and the anorexia of infection: potential mechanisms and treatments. Biological research for nursing. 2000;1(4):287-98.
52. Fruhbeck G. Intracellular signalling pathways activated by leptin. The Biochemical journal. 2006;393(Pt 1):7-20.
53. Scarlett JM, Jobst EE, Enriori PJ, Bowe DD, Batra AK, Grant WF, Cowley MA, Marks DL. Regulation of central melanocortin signaling by interleukin-1 beta. Endocrinology. 2007;148(9):4217-25.
54. Bierhaus A, Chen J, Liliensiek B, Nawroth PP. LPS and cytokine-activated endothelium. Semin Thromb Hemost. 2000;26(5):571-87.
55. Laflamme N, Lacroix S, Rivest S. An essential role of interleukin-1beta in mediating NF-kappaB activity and COX-2 transcription in cells of the blood-brain barrier in response to a systemic and localized inflammation but not during endotoxemia. J Neurosci. 1999;19(24):10923-30.
56. de Vries HE, Hoogendoorn KH, van Dijk J, Zijlstra FJ, van Dam AM, Breimer DD, van Berkel TJ, de Boer AG, Kuiper J. Eicosanoid production by rat cerebral endothelial cells: stimulation by lipopolysaccharide, interleukin-1 and interleukin-6. J Neuroimmunol. 1995;59(1-2):1-8.
57. Vitkovic L, Konsman JP, Bockaert J, Dantzer R, Homburger V, Jacque C. Cytokine signals propagate through the brain. Molecular psychiatry. 2000;5(6):604-15.
58. Plata-Salaman CR, Ilyin SE, Gayle D. Brain cytokine mRNAs in anorectic rats bearing prostate adenocarcinoma tumor cells. Am J Physiol. 1998;275(2 Pt 2):R566-73.
59. Strassmann G, Fong M, Kenney JS, Jacob CO. Evidence for the involvement of interleukin 6 in experimental cancer cachexia. J Clin Invest. 1992;89(5):1681-4.
60. Matthys P, Heremans H, Opdenakker G, Billiau A. Anti-interferon-gamma antibody treatment, growth of Lewis lung tumours in mice and tumour-associated cachexia. European journal of cancer. 1991;27(2):182-7.
61. Sabatini M, Yates AJ, Garrett IR, Chavez J, Dunn JF, Bonewald L, Mundy GR. Increased production of tumor necrosis factor by normal immune cells in a model of the humoral hypercalcemia of malignancy. Laboratory investigation; a journal of technical methods and pathology. 1990;63(5):676-82.
62. Torelli GF, Meguid MM, Moldawer LL, Edwards CK, 3rd, Kim HJ, Carter JL, Laviano A, Rossi Fanelli F. Use of recombinant human soluble TNF receptor in anorectic tumor-bearing rats. Am J Physiol. 1999;277(3 Pt 2):R850-5.
63. Fujita J, Tsujinaka T, Yano M, Ebisui C, Saito H, Katsume A, Akamatsu K, Ohsugi Y, Shiozaki H, Monden M. Anti-interleukin-6 receptor antibody prevents muscle atrophy in colon-26 adenocarcinoma-bearing mice with modulation of lysosomal and ATP-ubiquitin-dependent proteolytic pathways. International journal of cancer. 1996;68(5):637-43.
64. Sherry BA, Gelin J, Fong Y, Marano M, Wei H, Cerami A, Lowry SF, Lundholm KG, Moldawer LL. Anticachectin/tumor necrosis factor-alpha antibodies attenuate development of cachexia in tumor models. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 1989;3(8):1956-62.
65. Zhang GJ, Adachi I. Serum interleukin-6 levels correlate to tumor progression and prognosis in metastatic breast carcinoma. Anticancer research. 1999;19(2B):1427-32.
66. Wang YY, Lo GH, Lai KH, Cheng JS, Lin CK, Hsu PI. Increased serum concentrations of tumor necrosis factor-alpha are associated with disease progression and malnutrition in hepatocellular carcinoma. Journal of the Chinese Medical Association : JCMA. 2003;66(10):593-8.

67. Tazaki E, Shimizu N, Tanaka R, Yoshizumi M, Kamma H, Imoto S, Goya T, Kozawa K, Nishina A, Kimura H. Serum cytokine profiles in patients with prostate carcinoma. Experimental and therapeutic medicine. 2011;2(5):887-91.
68. Lewis AM, Varghese S, Xu H, Alexander HR. Interleukin-1 and cancer progression: the emerging role of interleukin-1 receptor antagonist as a novel therapeutic agent in cancer treatment. Journal of translational medicine. 2006;4:48.
69. Nakashima J, Tachibana M, Horiguchi Y, Oya M, Ohigashi T, Asakura H, Murai M. Serum interleukin 6 as a prognostic factor in patients with prostate cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2000;6(7):2702-6.
70. Brown DA, Ward RL, Buckhaults P, Liu T, Romans KE, Hawkins NJ, Bauskin AR, Kinzler KW, Vogelstein B, Breit SN. MIC-1 serum level and genotype: associations with progress and prognosis of colorectal carcinoma. Clinical cancer research : an official journal of the American Association for Cancer Research. 2003;9(7):2642-50.
71. Licinio J, Wong ML. Pathways and mechanisms for cytokine signaling of the central nervous system. The Journal of clinical investigation. 1997;100(12):2941-7.
72. Plata-Salaman CR. Central nervous system mechanisms contributing to the cachexia-anorexia syndrome. Nutrition. 2000;16(10):1009-12.
73. Mueller TC, Bachmann J, Prokopchuk O, Friess H, Martignoni ME. Molecular pathways leading to loss of skeletal muscle mass in cancer cachexia--can findings from animal models be translated to humans? BMC cancer. 2016;16:75.
74. Cao PR, Kim HJ, Lecker SH. Ubiquitin-protein ligases in muscle wasting. The international journal of biochemistry & cell biology. 2005;37(10):2088-97.
75. Burckart K, Beca S, Urban RJ, Sheffield-Moore M. Pathogenesis of muscle wasting in cancer cachexia: targeted anabolic and anticatabolic therapies. Curr Opin Clin Nutr Metab Care. 2010;13(4):410-6.
76. Williams A, Sun X, Fischer JE, Hasselgren PO. The expression of genes in the ubiquitin-proteasome proteolytic pathway is increased in skeletal muscle from patients with cancer. Surgery. 1999;126(4):744-9; discussion 9-50.
77. Yuan L, Han J, Meng Q, Xi Q, Zhuang Q, Jiang Y, Han Y, Zhang B, Fang J, Wu G. Muscle-specific E3 ubiquitin ligases are involved in muscle atrophy of cancer cachexia: an in vitro and in vivo study. Oncology reports. 2015;33(5):2261-8.
78. Gomes MD, Lecker SH, Jagoe RT, Navon A, Goldberg AL. Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy. Proceedings of the National Academy of Sciences of the United States of America. 2001;98(25):14440-5.
79. Chen JA, Splenser A, Guillory B, Luo J, Mendiratta M, Belinova B, Halder T, Zhang G, Li YP, Garcia JM. Ghrelin prevents tumour- and cisplatin-induced muscle wasting: characterization of multiple mechanisms involved. Journal of cachexia, sarcopenia and muscle. 2015;6(2):132-43.
80. Banduseela V, Ochala J, Lamberg K, Kalimo H, Larsson L. Muscle paralysis and myosin loss in a patient with cancer cachexia. Acta myologica : myopathies and cardiomyopathies : official journal of the Mediterranean Society of Myology. 2007;26(3):136-44.
81. Zhou X, Wang JL, Lu J, Song Y, Kwak KS, Jiao Q, Rosenfeld R, Chen Q, Boone T, Simonet WS, Lacey DL, Goldberg AL, Han HQ. Reversal of cancer cachexia and muscle wasting by ActRIIB antagonism leads to prolonged survival. Cell. 2010;142(4):531-43.
82. Zhang L, Tang H, Kou Y, Li R, Zheng Y, Wang Q, Zhou X, Jin L. MG132-mediated inhibition of the ubiquitin-proteasome pathway ameliorates cancer cachexia. Journal of cancer research and clinical oncology. 2013;139(7):1105-15.

83. Price CJ, Hoyda TD, Ferguson AV. The area postrema: a brain monitor and integrator of systemic autonomic state. The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry. 2008;14(2):182-94.
84. Johnson AK, Gross PM. Sensory circumventricular organs and brain homeostatic pathways. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 1993;7(8):678-86.
85. Johnson AK, Gross PM. Sensory circumventricular organs and brain homeostatic pathways. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 1993;7(8):678-86.
86. McKinley MJ, McAllen RM, Davern P, Giles ME, Penschow J, Sunn N, Uschakov A, Oldfield BJ. The sensory circumventricular organs of the mammalian brain. Adv Anat Embryol Cell Biol. 2003;172:III-XII, 1-122, back cover.
87. Price CJ, Hoyda TD, Ferguson AV. The area postrema: a brain monitor and integrator of systemic autonomic state. Neuroscientist. 2008;14(2):182-94.
88. Shapiro RE, Miselis RR. The central neural connections of the area postrema of the rat. J Comp Neurol. 1985;234(3):344-64.
89. Butler PC, Chou J, Carter WB, Wang YN, Bu BH, Chang D, Chang JK, Rizza RA. Effects of meal ingestion on plasma amylin concentration in NIDDM and nondiabetic humans. Diabetes. 1990;39(6):752-6.
90. Moore CX, Cooper GJ. Co-secretion of amylin and insulin from cultured islet beta-cells: modulation by nutrient secretagogues, islet hormones and hypoglycemic agents. Biochem Biophys Res Commun. 1991;179(1):1-9.
91. Potes CS, Lutz TA. Brainstem mechanisms of amylin-induced anorexia. Physiol Behav. 2010;100(5):511-8.
92. Miller AD, Leslie RA. The area postrema and vomiting. Front Neuroendocrinol. 1994;15(4):301-20.
93. Borison HL, Brizzee KR. Morphology of emetic chemoreceptor trigger zone in cat medulla oblongata. Proc Soc Exp Biol Med. 1951;77(1):38-42.
94. Zuger D, Forster K, Lutz TA, Riediger T. Amylin and GLP-1 target different populations of area postrema neurons that are both modulated by nutrient stimuli. Physiol Behav. 2013;112-113:61-9.
95. Miller AD, Leslie RA. The area postrema and vomiting. Frontiers in neuroendocrinology. 1994;15(4):301-20.
96. Lindstrom PA, Brizzee KR. Relief of intractable vomiting from surgical lesions in the area postrema. Journal of neurosurgery. 1962;19:228-36.
97. Brady LS, Lynn AB, Herkenham M, Gottesfeld Z. Systemic interleukin-1 induces early and late patterns of c-fos mRNA expression in brain. J Neurosci. 1994;14(8):4951-64.
98. Sagar SM, Price KJ, Kasting NW, Sharp FR. Anatomic patterns of Fos immunostaining in rat brain following systemic endotoxin administration. Brain Res Bull. 1995;36(4):381-92.
99. Johnen H, Lin S, Kuffner T, Brown DA, Tsai VW, Bauskin AR, Wu L, Pankhurst G, Jiang L, Junankar S, Hunter M, Fairlie WD, Lee NJ, Enriquez RF, Baldock PA, Corey E, Apple FS, Murakami MM, Lin EJ, Wang C, During MJ, Sainsbury A, Herzog H, Breit SN. Tumor-induced anorexia and weight loss are mediated by the TGF-beta superfamily cytokine MIC-1. Nat Med. 2007;13(11):1333-40.
100. Bernstein IL, Taylor EM, Bentson KL. TNF-induced anorexia and learned food aversions are attenuated by area postrema lesions. Am J Physiol. 1991;260(5 Pt 2):R906-10.

101. Tsai VW, Manandhar R, Jorgensen SB, Lee-Ng KK, Zhang HP, Marquis CP, Jiang L, Husaini Y, Lin S, Sainsbury A, Sawchenko PE, Brown DA, Breit SN. The anorectic actions of the TGFbeta cytokine MIC-1/GDF15 require an intact brainstem area postrema and nucleus of the solitary tract. PloS one. 2014;9(6):e100370.
102. Ricardo JA, Koh ET. Anatomical evidence of direct projections from the nucleus of the solitary tract to the hypothalamus, amygdala, and other forebrain structures in the rat. Brain Res. 1978;153(1):1-26.
103. Vigier D, Rouviere A. Afferent and efferent connections of the area postrema demonstrated by the horseradish peroxidase method. Arch Ital Biol. 1979;117(4):325-39.
104. Grill HJ, Hayes MR. Hindbrain neurons as an essential hub in the neuroanatomically distributed control of energy balance. Cell Metab. 2012;16(3):296-309.
105. Ricardo JA, Koh ET. Anatomical evidence of direct projections from the nucleus of the solitary tract to the hypothalamus, amygdala, and other forebrain structures in the rat. Brain Res. 1978;153(1):1-26.
106. Bernstein IL. Taste aversion learning: a contemporary perspective. Nutrition. 1999;15(3):229-34.
107. Sugiyama Y, Suzuki T, DeStefino VJ, Yates BJ. Integrative responses of neurons in nucleus tractus solitarius to visceral afferent stimulation and vestibular stimulation in vertical planes. Am J Physiol Regul Integr Comp Physiol. 2011;301(5):R1380-90.
108. Horn CC. Measuring the nausea-to-emesis continuum in non-human animals: refocusing on gastrointestinal vagal signaling. Experimental brain research. 2014;232(8):2471-81.
109. Niijima A, Yamamoto T. The effects of lithium chloride on the activity of the afferent nerve fibers from the abdominal visceral organs in the rat. Brain research bulletin. 1994;35(2):141-5.
110. Yamamoto T, Shimura T, Sako N, Azuma S, Bai WZ, Wakisaka S. C-fos expression in the rat brain after intraperitoneal injection of lithium chloride. Neuroreport. 1992;3(12):1049-52.
111. Ruud J, Blomqvist A. Identification of rat brainstem neuronal structures activated during cancer-induced anorexia. J Comp Neurol. 2007;504(3):275-86.
112. Bernstein IL, Treneer CM, Kott JN. Area postrema mediates tumor effects on food intake, body weight, and learned aversions. Am J Physiol. 1985;249(3 Pt 2):R296-300.
113. Asarian L, Geary N. Sex differences in the physiology of eating. American journal of physiology Regulatory, integrative and comparative physiology. 2013;305(11):R1215-67.
114. Lutz TA, Senn M, Althaus J, Del Prete E, Ehrensperger F, Scharrer E. Lesion of the area postrema/nucleus of the solitary tract (AP/NTS) attenuates the anorectic effects of amylin and calcitonin gene-related peptide (CGRP) in rats. Peptides. 1998;19(2):309-17.
115. Holst JJ. The physiology of glucagon-like peptide 1. Physiol Rev. 2007;87(4):1409-39.
116. Sandoval DA, D'Alessio DA. Physiology of proglucagon peptides: role of glucagon and GLP-1 in health and disease. Physiol Rev. 2015;95(2):513-48.
117. de Heer J, Rasmussen C, Coy DH, Holst JJ. Glucagon-like peptide-1, but not glucose-dependent insulinotropic peptide, inhibits glucagon secretion via somatostatin (receptor subtype 2) in the perfused rat pancreas. Diabetologia. 2008;51(12):2263-70.
118. Elliott RM, Morgan LM, Tredger JA, Deacon S, Wright J, Marks V. Glucagon-like peptide-1 (7-36)amide and glucose-dependent insulinotropic polypeptide secretion in

response to nutrient ingestion in man: acute post-prandial and 24-h secretion patterns. J Endocrinol. 1993;138(1):159-66.
119. Baggio LL, Drucker DJ. Biology of incretins: GLP-1 and GIP. Gastroenterology. 2007;132(6):2131-57.
120. Steinert RE, Beglinger C, Langhans W. Intestinal GLP-1 and satiation: from man to rodents and back. Int J Obes (Lond). 2016;40(2):198-205.
121. Hayes MR, De Jonghe BC, Kanoski SE. Role of the glucagon-like-peptide-1 receptor in the control of energy balance. Physiol Behav. 2010;100(5):503-10.
122. Hayes MR. Neuronal and intracellular signaling pathways mediating GLP-1 energy balance and glycemic effects. Physiol Behav. 2012;106(3):413-6.
123. Turton MD, O'Shea D, Gunn I, Beak SA, Edwards CM, Meeran K, Choi SJ, Taylor GM, Heath MM, Lambert PD, Wilding JP, Smith DM, Ghatei MA, Herbert J, Bloom SR. A role for glucagon-like peptide-1 in the central regulation of feeding. Nature. 1996;379(6560):69-72.
124. Williams KW, Elmquist JK. From neuroanatomy to behavior: central integration of peripheral signals regulating feeding behavior. Nat Neurosci. 2012;15(10):1350-5.
125. Punjabi M, Arnold M, Ruttimann E, Graber M, Geary N, Pacheco-Lopez G, Langhans W. Circulating glucagon-like peptide-1 (GLP-1) inhibits eating in male rats by acting in the hindbrain and without inducing avoidance. Endocrinology. 2014;155(5):1690-9.
126. Mayo KE, Miller LJ, Bataille D, Dalle S, Goke B, Thorens B, Drucker DJ. International Union of Pharmacology. XXXV. The glucagon receptor family. Pharmacol Rev. 2003;55(1):167-94.
127. Merchenthaler I, Lane M, Shughrue P. Distribution of pre-pro-glucagon and glucagon-like peptide-1 receptor messenger RNAs in the rat central nervous system. J Comp Neurol. 1999;403(2):261-80.
128. Wei Y, Mojsov S. Distribution of GLP-1 and PACAP receptors in human tissues. Acta Physiol Scand. 1996;157(3):355-7.
129. Dunphy JL, Taylor RG, Fuller PJ. Tissue distribution of rat glucagon receptor and GLP-1 receptor gene expression. Mol Cell Endocrinol. 1998;141(1-2):179-86.
130. Shimizu I, Hirota M, Ohboshi C, Shima K. Identification and localization of glucagon-like peptide-1 and its receptor in rat brain. Endocrinology. 1987;121(3):1076-82.
131. Llewellyn-Smith IJ, Reimann F, Gribble FM, Trapp S. Preproglucagon neurons project widely to autonomic control areas in the mouse brain. Neuroscience. 2011;180:111-21.
132. Cork SC, Richards JE, Holt MK, Gribble FM, Reimann F, Trapp S. Distribution and characterisation of Glucagon-like peptide-1 receptor expressing cells in the mouse brain. Molecular metabolism. 2015;4(10):718-31.
133. Jin SL, Han VK, Simmons JG, Towle AC, Lauder JM, Lund PK. Distribution of glucagonlike peptide I (GLP-I), glucagon, and glicentin in the rat brain: an immunocytochemical study. J Comp Neurol. 1988;271(4):519-32.
134. Garber AJ. Long-acting glucagon-like peptide 1 receptor agonists: a review of their efficacy and tolerability. Diabetes Care. 2011;34 Suppl 2:S279-84.
135. Marre M, Shaw J, Brandle M, Bebakar WM, Kamaruddin NA, Strand J, Zdravkovic M, Le Thi TD, Colagiuri S. Liraglutide, a once-daily human GLP-1 analogue, added to a sulphonylurea over 26 weeks produces greater improvements in glycaemic and weight control compared with adding rosiglitazone or placebo in subjects with Type 2 diabetes (LEAD-1 SU). Diabet Med. 2009;26(3):268-78.

136. Calara F, Taylor K, Han J, Zabala E, Carr EM, Wintle M, Fineman M. A randomized, open-label, crossover study examining the effect of injection site on bioavailability of exenatide (synthetic exendin-4). Clin Ther. 2005;27(2):210-5.
137. Thiele TE, Seeley RJ, D'Alessio D, Eng J, Bernstein IL, Woods SC, van Dijk G. Central infusion of glucagon-like peptide-1-(7-36) amide (GLP-1) receptor antagonist attenuates lithium chloride-induced c-Fos induction in rat brainstem. Brain Res. 1998;801(1-2):164-70.
138. Van Dijk G, Thiele TE, Donahey JC, Campfield LA, Smith FJ, Burn P, Bernstein IL, Woods SC, Seeley RJ. Central infusions of leptin and GLP-1-(7-36) amide differentially stimulate c-FLI in the rat brain. Am J Physiol. 1996;271(4 Pt 2):R1096-100.
139. Kinzig KP, D'Alessio DA, Seeley RJ. The diverse roles of specific GLP-1 receptors in the control of food intake and the response to visceral illness. J Neurosci. 2002;22(23):10470-6.
140. Seeley RJ, Blake K, Rushing PA, Benoit S, Eng J, Woods SC, D'Alessio D. The role of CNS glucagon-like peptide-1 (7-36) amide receptors in mediating the visceral illness effects of lithium chloride. J Neurosci. 2000;20(4):1616-21.
141. Rinaman L. A functional role for central glucagon-like peptide-1 receptors in lithium chloride-induced anorexia. Am J Physiol. 1999;277(5 Pt 2):R1537-40.
142. De Jonghe BC, Holland RA, Olivos DR, Rupprecht LE, Kanoski SE, Hayes MR. Hindbrain GLP-1 receptor mediation of cisplatin-induced anorexia and nausea. Physiol Behav. 2016;153:109-14.
143. Rinaman L. Interoceptive stress activates glucagon-like peptide-1 neurons that project to the hypothalamus. Am J Physiol. 1999;277(2 Pt 2):R582-90.
144. Grill HJ, Carmody JS, Amanda Sadacca L, Williams DL, Kaplan JM. Attenuation of lipopolysaccharide anorexia by antagonism of caudal brain stem but not forebrain GLP-1-R. Am J Physiol Regul Integr Comp Physiol. 2004;287(5):R1190-3.
145. Bauskin AR, Brown DA, Kuffner T, Johnen H, Luo XW, Hunter M, Breit SN. Role of macrophage inhibitory cytokine-1 in tumorigenesis and diagnosis of cancer. Cancer research. 2006;66(10):4983-6.
146. Staff AC, Bock AJ, Becker C, Kempf T, Wollert KC, Davidson B. Growth differentiation factor-15 as a prognostic biomarker in ovarian cancer. Gynecologic oncology. 2010;118(3):237-43.
147. Welsh JB, Sapinoso LM, Kern SG, Brown DA, Liu T, Bauskin AR, Ward RL, Hawkins NJ, Quinn DI, Russell PJ, Sutherland RL, Breit SN, Moskaluk CA, Frierson HF, Jr., Hampton GM. Large-scale delineation of secreted protein biomarkers overexpressed in cancer tissue and serum. Proceedings of the National Academy of Sciences of the United States of America. 2003;100(6):3410-5.
148. Zimmers TA, Jin X, Gutierrez JC, Acosta C, McKillop IH, Pierce RH, Koniaris LG. Effect of in vivo loss of GDF-15 on hepatocellular carcinogenesis. Journal of cancer research and clinical oncology. 2008;134(7):753-9.
149. Skipworth RJ, Deans DA, Tan BH, Sangster K, Paterson-Brown S, Brown DA, Hunter M, Breit SN, Ross JA, Fearon KC. Plasma MIC-1 correlates with systemic inflammation but is not an independent determinant of nutritional status or survival in oesophago-gastric cancer. British journal of cancer. 2010;102(4):665-72.
150. Koopmann J, Buckhaults P, Brown DA, Zahurak ML, Sato N, Fukushima N, Sokoll LJ, Chan DW, Yeo CJ, Hruban RH, Breit SN, Kinzler KW, Vogelstein B, Goggins M. Serum macrophage inhibitory cytokine 1 as a marker of pancreatic and other periampullary cancers. Clinical cancer research : an official journal of the American Association for Cancer Research. 2004;10(7):2386-92.

151. Macia L, Tsai VW, Nguyen AD, Johnen H, Kuffner T, Shi YC, Lin S, Herzog H, Brown DA, Breit SN, Sainsbury A. Macrophage inhibitory cytokine 1 (MIC-1/GDF15) decreases food intake, body weight and improves glucose tolerance in mice on normal & obesogenic diets. PloS one. 2012;7(4):e34868.
152. Tsai VW, Macia L, Johnen H, Kuffner T, Manadhar R, Jorgensen SB, Lee-Ng KK, Zhang HP, Wu L, Marquis CP, Jiang L, Husaini Y, Lin S, Herzog H, Brown DA, Sainsbury A, Breit SN. TGF-b superfamily cytokine MIC-1/GDF15 is a physiological appetite and body weight regulator. PloS one. 2013;8(2):e55174.
153. Suzuki K, Simpson KA, Minnion JS, Shillito JC, Bloom SR. The role of gut hormones and the hypothalamus in appetite regulation. Endocr J. 2010;57(5):359-72.
154. Chronwall BM. Anatomy and physiology of the neuroendocrine arcuate nucleus. Peptides. 1985;6 Suppl 2:1-11.
155. Broberger C, De Lecea L, Sutcliffe JG, Hokfelt T. Hypocretin/orexin- and melanin-concentrating hormone-expressing cells form distinct populations in the rodent lateral hypothalamus: relationship to the neuropeptide Y and agouti gene-related protein systems. J Comp Neurol. 1998;402(4):460-74.
156. Broberger C, Landry M, Wong H, Walsh JN, Hokfelt T. Subtypes Y1 and Y2 of the neuropeptide Y receptor are respectively expressed in pro-opiomelanocortin- and neuropeptide-Y-containing neurons of the rat hypothalamic arcuate nucleus. Neuroendocrinology. 1997;66(6):393-408.
157. Fan W, Boston BA, Kesterson RA, Hruby VJ, Cone RD. Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. Nature. 1997;385(6612):165-8.
158. Ollmann MM, Wilson BD, Yang YK, Kerns JA, Chen Y, Gantz I, Barsh GS. Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. Science. 1997;278(5335):135-8.
159. Stanley BG, Kyrkouli SE, Lampert S, Leibowitz SF. Neuropeptide Y chronically injected into the hypothalamus: a powerful neurochemical inducer of hyperphagia and obesity. Peptides. 1986;7(6):1189-92.
160. Roseberry AG, Liu H, Jackson AC, Cai X, Friedman JM. Neuropeptide Y-mediated inhibition of proopiomelanocortin neurons in the arcuate nucleus shows enhanced desensitization in ob/ob mice. Neuron. 2004;41(5):711-22.
161. Woods SC, D'Alessio DA. Central control of body weight and appetite. J Clin Endocrinol Metab. 2008;93(11 Suppl 1):S37-50.
162. Luquet S, Magnan C. The central nervous system at the core of the regulation of energy homeostasis. Front Biosci (Schol Ed). 2009;1:448-65.
163. McCarthy HD, McKibbin PE, Perkins AV, Linton EA, Williams G. Alterations in hypothalamic NPY and CRF in anorexic tumor-bearing rats. Am J Physiol. 1993;264(4 Pt 1):E638-43.
164. Chance WT, Balasubramaniam A, Dayal R, Brown J, Fischer JE. Hypothalamic concentration and release of neuropeptide Y into microdialysates is reduced in anorectic tumor-bearing rats. Life sciences. 1994;54(24):1869-74.
165. Chance WT, Balasubramaniam A, Thompson H, Mohapatra B, Ramo J, Fischer JE. Assessment of feeding response of tumor-bearing rats to hypothalamic injection and infusion of neuropeptide Y. Peptides. 1996;17(5):797-801.
166. Wisse BE, Frayo RS, Schwartz MW, Cummings DE. Reversal of cancer anorexia by blockade of central melanocortin receptors in rats. Endocrinology. 2001;142(8):3292-301.
167. Weyermann P, Dallmann R, Magyar J, Anklin C, Hufschmid M, Dubach-Powell J, Courdier-Fruh I, Hennebohle M, Nordhoff S, Mondadori C. Orally available selective

melanocortin-4 receptor antagonists stimulate food intake and reduce cancer-induced cachexia in mice. PloS one. 2009;4(3):e4774.
168. Joppa MA, Gogas KR, Foster AC, Markison S. Central infusion of the melanocortin receptor antagonist agouti-related peptide (AgRP(83-132)) prevents cachexia-related symptoms induced by radiation and colon-26 tumors in mice. Peptides. 2007;28(3):636-42.
169. Sonti G, Ilyin SE, Plata-Salaman CR. Neuropeptide Y blocks and reverses interleukin-1 beta-induced anorexia in rats. Peptides. 1996;17(3):517-20.
170. Gayle D, Ilyin SE, Plata-Salaman CR. Central nervous system IL-1 beta system and neuropeptide Y mRNAs during IL-1 beta-induced anorexia in rats. Brain research bulletin. 1997;44(3):311-7.
171. Grossberg AJ, Scarlett JM, Zhu X, Bowe DD, Batra AK, Braun TP, Marks DL. Arcuate nucleus proopiomelanocortin neurons mediate the acute anorectic actions of leukemia inhibitory factor via gp130. Endocrinology. 2010;151(2):606-16.
172. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature. 1999;402(6762):656-60.
173. Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, Matsukura S, Kangawa K, Nakazato M. Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. Endocrinology. 2000;141(11):4255-61.
174. Inui A, Asakawa A, Bowers CY, Mantovani G, Laviano A, Meguid MM, Fujimiya M. Ghrelin, appetite, and gastric motility: the emerging role of the stomach as an endocrine organ. Faseb J. 2004;18(3):439-56.
175. Zhang JV, Ren PG, Avsian-Kretchmer O, Luo CW, Rauch R, Klein C, Hsueh AJ. Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. Science. 2005;310(5750):996-9.
176. Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE, Weigle DS. A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. Diabetes. 2001;50(8):1714-9.
177. Tschop M, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. Nature. 2000;407(6806):908-13.
178. Asakawa A, Inui A, Kaga T, Yuzuriha H, Nagata T, Ueno N, Makino S, Fujimiya M, Niijima A, Fujino MA, Kasuga M. Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. Gastroenterology. 2001;120(2):337-45.
179. Abou Heif HM, Deif MM, Abdel Aziz HK. Effect of food restriction on ghrelin in adult male rats and its relation to male reproductive hormones. Andrologia. 2010;42(2):97-105.
180. Vestergaard ET, Hansen TK, Gormsen LC, Jakobsen P, Moller N, Christiansen JS, Jorgensen JO. Constant intravenous ghrelin infusion in healthy young men: clinical pharmacokinetics and metabolic effects. American journal of physiology Endocrinology and metabolism. 2007;292(6):E1829-36.
181. Akamizu T, Takaya K, Irako T, Hosoda H, Teramukai S, Matsuyama A, Tada H, Miura K, Shimizu A, Fukushima M, Yokode M, Tanaka K, Kangawa K. Pharmacokinetics, safety, and endocrine and appetite effects of ghrelin administration in young healthy subjects. European journal of endocrinology. 2004;150(4):447-55.
182. Hosoda H, Kangawa K. Standard sample collections for blood ghrelin measurements. Methods in enzymology. 2012;514:113-26.

183. Guan X, Yu H, Palyha O, McKee K, Feighner S, Sirinathsinghji D, Smith R, Van der Ploeg L, Howard A. Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. Brain Res Mol Brain Res. 1997;48(1):23-9.
184. Willesen M, Kristensen P, Romer J. Co-localization of growth hormone secretagogue receptor and NPY mRNA in the arcuate nucleus of the rat. Neuroendocrinology. 1999;70(5):306-16.
185. Willesen MG, Kristensen P, Romer J. Co-localization of growth hormone secretagogue receptor and NPY mRNA in the arcuate nucleus of the rat. Neuroendocrinology. 1999;70(5):306-16.
186. Wang L, Saint-Pierre DH, Tache Y. Peripheral ghrelin selectively increases Fos expression in neuropeptide Y - synthesizing neurons in mouse hypothalamic arcuate nucleus. Neurosci Lett. 2002;325(1):47-51.
187. Goto M, Arima H, Watanabe M, Hayashi M, Banno R, Sato I, Nagasaki H, Oiso Y. Ghrelin increases neuropeptide Y and agouti-related peptide gene expression in the arcuate nucleus in rat hypothalamic organotypic cultures. Endocrinology. 2006;147(11):5102-9.
188. Traebert M, Riediger T, Whitebread S, Scharrer E, Schmid HA. Ghrelin acts on leptin-responsive neurones in the rat arcuate nucleus. J Neuroendocrinol. 2002;14(7):580-6.
189. Riediger T, Traebert M, Schmid HA, Scheel C, Lutz TA, Scharrer E. Site-specific effects of ghrelin on the neuronal activity in the hypothalamic arcuate nucleus. Neurosci Lett. 2003;341(2):151-5.
190. Cowley MA, Smith RG, Diano S, Tschop M, Pronchuk N, Grove KL, Strasburger CJ, Bidlingmaier M, Esterman M, Heiman ML, Garcia-Segura LM, Nillni EA, Mendez P, Low MJ, Sotonyi P, Friedman JM, Liu H, Pinto S, Colmers WF, Cone RD, Horvath TL. The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. Neuron. 2003;37(4):649-61.
191. Chen HY, Trumbauer ME, Chen AS, Weingarth DT, Adams JR, Frazier EG, Shen Z, Marsh DJ, Feighner SD, Guan XM, Ye Z, Nargund RP, Smith RG, Van der Ploeg LH, Howard AD, MacNeil DJ, Qian S. Orexigenic action of peripheral ghrelin is mediated by neuropeptide Y and agouti-related protein. Endocrinology. 2004;145(6):2607-12.
192. Theander-Carrillo C, Wiedmer P, Cettour-Rose P, Nogueiras R, Perez-Tilve D, Pfluger P, Castaneda TR, Muzzin P, Schurmann A, Szanto I, Tschop MH, Rohner-Jeanrenaud F. Ghrelin action in the brain controls adipocyte metabolism. J Clin Invest. 2006;116(7):1983-93.
193. Sangiao-Alvarellos S, Vazquez MJ, Varela L, Nogueiras R, Saha AK, Cordido F, Lopez M, Dieguez C. Central ghrelin regulates peripheral lipid metabolism in a growth hormone-independent fashion. Endocrinology. 2009;150(10):4562-74.
194. Yasuda T, Masaki T, Kakuma T, Yoshimatsu H. Centrally administered ghrelin suppresses sympathetic nerve activity in brown adipose tissue of rats. Neurosci Lett. 2003;349(2):75-8.
195. Tsubone T, Masaki T, Katsuragi I, Tanaka K, Kakuma T, Yoshimatsu H. Ghrelin regulates adiposity in white adipose tissue and UCP1 mRNA expression in brown adipose tissue in mice. Regulatory peptides. 2005;130(1-2):97-103.
196. Broglio F, Arvat E, Benso A, Gottero C, Muccioli G, Papotti M, van der Lely AJ, Deghenghi R, Ghigo E. Ghrelin, a natural GH secretagogue produced by the stomach, induces hyperglycemia and reduces insulin secretion in humans. J Clin Endocrinol Metab. 2001;86(10):5083-6.

197. Tong J, Prigeon RL, Davis HW, Bidlingmaier M, Kahn SE, Cummings DE, Tschop MH, D'Alessio D. Ghrelin suppresses glucose-stimulated insulin secretion and deteriorates glucose tolerance in healthy humans. Diabetes. 2010;59(9):2145-51.
198. Dixit VD, Schaffer EM, Pyle RS, Collins GD, Sakthivel SK, Palaniappan R, Lillard JW, Jr., Taub DD. Ghrelin inhibits leptin- and activation-induced proinflammatory cytokine expression by human monocytes and T cells. The Journal of clinical investigation. 2004;114(1):57-66.
199. Li WG, Gavrila D, Liu X, Wang L, Gunnlaugsson S, Stoll LL, McCormick ML, Sigmund CD, Tang C, Weintraub NL. Ghrelin inhibits proinflammatory responses and nuclear factor-kappaB activation in human endothelial cells. Circulation. 2004;109(18):2221-6.
200. Wren AM, Small CJ, Ward HL, Murphy KG, Dakin CL, Taheri S, Kennedy AR, Roberts GH, Morgan DG, Ghatei MA, Bloom SR. The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. Endocrinology. 2000;141(11):4325-8.
201. Schiaffino S, Mammucari C. Regulation of skeletal muscle growth by the IGF1-Akt/PKB pathway: insights from genetic models. Skeletal muscle. 2011;1(1):4.
202. Garcia JM, Polvino WJ. Pharmacodynamic hormonal effects of anamorelin, a novel oral ghrelin mimetic and growth hormone secretagogue in healthy volunteers. Growth Horm IGF Res. 2009;19(3):267-73.
203. Takaya K, Ariyasu H, Kanamoto N, Iwakura H, Yoshimoto A, Harada M, Mori K, Komatsu Y, Usui T, Shimatsu A, Ogawa Y, Hosoda K, Akamizu T, Kojima M, Kangawa K, Nakao K. Ghrelin strongly stimulates growth hormone release in humans. The Journal of clinical endocrinology and metabolism. 2000;85(12):4908-11.
204. Garcia JM, Garcia-Touza M, Hijazi RA, Taffet G, Epner D, Mann D, Smith RG, Cunningham GR, Marcelli M. Active ghrelin levels and active to total ghrelin ratio in cancer-induced cachexia. The Journal of clinical endocrinology and metabolism. 2005;90(5):2920-6.
205. Temel JS, Abernethy AP, Currow DC, Friend J, Duus EM, Yan Y, Fearon KC. Anamorelin in patients with non-small-cell lung cancer and cachexia (ROMANA 1 and ROMANA 2): results from two randomised, double-blind, phase 3 trials. Lancet Oncol. 2016;17(4):519-31.
206. Karasawa H, Pietra C, Giuliano C, Garcia-Rubio S, Xu X, Yakabi S, Tache Y, Wang L. New ghrelin agonist, HM01 alleviates constipation and L-dopa-delayed gastric emptying in 6-hydroxydopamine rat model of Parkinson's disease. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society. 2014;26(12):1771-82.
207. Gordon JN, Green SR, Goggin PM. Cancer cachexia. Qjm. 2005;98(11):779-88.
208. Tisdale MJ. Biology of cachexia. J Natl Cancer Inst. 1997;89(23):1763-73.
209. Borner T, Arnold M, Ruud J, Breit SN, Langhans W, Lutz TA, Blomqvist A, Riediger T. Anorexia-cachexia syndrome in hepatoma tumour-bearing rats requires the area postrema but not vagal afferents and is paralleled by increased MIC-1/GDF15. Journal of cachexia, sarcopenia and muscle. 2016.
210. Eissele R, Goke R, Willemer S, Harthus HP, Vermeer H, Arnold R, Goke B. Glucagon-like peptide-1 cells in the gastrointestinal tract and pancreas of rat, pig and man. Eur J Clin Invest. 1992;22(4):283-91.
211. Hansen CF, Vrang N, Sangild PT, Jelsing J. Novel insight into the distribution of L-cells in the rat intestinal tract. Am J Transl Res. 2013;5(3):347-58.

212. Grill HJ, Hayes MR. Hindbrain Neurons as an Essential Hub in the Neuroanatomically Distributed Control of Energy Balance. Cell Metab. 2012;16(3):296-309.
213. Ruud J, Blomqvist A. Identification of rat brainstem neuronal structures activated during cancer-induced anorexia. J Comp Neurol. 2007;504(3):275-86.
214. Endo T, Minami M, Nakayasu M, Hirafuji M, Hamaue N, Omae N, Kang YN, Iwanaga T. Effects of granisetron and vagotomy on c-fos mRNA expression in the rat medulla oblongata as assessed by in situ hybridization. Biomed Res-Tokyo. 2004;25(5):229-35.
215. Horn CC, Ciucci M, Chaudhury A. Brain Fos expression during 48 h after cisplatin treatment: neural pathways for acute and delayed visceral sickness. Autonomic neuroscience : basic & clinical. 2007;132(1-2):44-51.
216. Yamamoto T, Nagai T, Shimura T, Yasoshima Y. Roles of chemical mediators in the taste system. Japanese journal of pharmacology. 1998;76(4):325-48.
217. Mele PC, McDonough JR, McLean DB, O'Halloran KP. Cisplatin-induced conditioned taste aversion: attenuation by dexamethasone but not zacopride or GR38032F. European journal of pharmacology. 1992;218(2-3):229-36.
218. Paxinos G, Watson C. The Rat Brain in Stereotactic Coordinates. 6th Edition ed. Elsevier; 2006.
219. Ritter RC, Slusser PG, Stone S. Glucoreceptors controlling feeding and blood glucose: location in the hindbrain. Science. 1981;213(4506):451-2.
220. Borner T, Loi L, Pietra C, Giuliano C, Lutz TA, Riediger T. The ghrelin receptor agonist HM01 mimics the neuronal effects of ghrelin in the arcuate nucleus and attenuates anorexia-cachexia syndrome in tumor-bearing rats. Am J Physiol Regul Integr Comp Physiol. 2016;311(1):R89-96.
221. Barrera JG, Jones KR, Herman JP, D'Alessio DA, Woods SC, Seeley RJ. Hyperphagia and increased fat accumulation in two models of chronic CNS glucagon-like peptide-1 loss of function. J Neurosci. 2011;31(10):3904-13.
222. Larsen PJ, Tang-Christensen M, Holst JJ, Orskov C. Distribution of glucagon-like peptide-1 and other preproglucagon-derived peptides in the rat hypothalamus and brainstem. Neuroscience. 1997;77(1):257-70.
223. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-8.
224. Zhao S, Kanoski SE, Yan J, Grill HJ, Hayes MR. Hindbrain leptin and glucagon-like-peptide-1 receptor signaling interact to suppress food intake in an additive manner. Int J Obes (Lond). 2012;36(12):1522-8.
225. Hayes MR, Bradley L, Grill HJ. Endogenous Hindbrain Glucagon-Like Peptide-1 Receptor Activation Contributes to the Control of Food Intake by Mediating Gastric Satiation Signaling. Endocrinology. 2009;150(6):2654-9.
226. Ruttimann EB, Arnold M, Hillebrand JJ, Geary N, Langhans W. Intrameal hepatic portal and intraperitoneal infusions of glucagon-like peptide-1 reduce spontaneous meal size in the rat via different mechanisms. Endocrinology. 2009;150(3):1174-81.
227. Krieger JP, Langhans W, Lee SJ. Vagal mediation of GLP-1's effects on food intake and glycemia. Physiol Behav. 2015;152(Pt B):372-80.
228. Punjabi M, Arnold M, Geary N, Langhans W, Pacheco-Lopez G. Peripheral glucagon-like peptide-1 (GLP-1) and satiation. Physiol Behav. 2011;105(1):71-6.
229. Krieger JP, Arnold M, Pettersen KG, Lossel P, Langhans W, Lee SJ. Knockdown of GLP-1 Receptors in Vagal Afferents Affects Normal Food Intake and Glycemia. Diabetes. 2016;65(1):34-43.

230. Daniels D, Marshall A. Evaluating the potential for rostral diffusion in the cerebral ventricles using angiotensin II-induced drinking in rats. Brain Res. 2012;1486:62-7.
231. Prasad-Reddy L, Isaacs D. A clinical review of GLP-1 receptor agonists: efficacy and safety in diabetes and beyond. Drugs in context. 2015;4:212283.
232. Tomas E, Stanojevic V, McManus K, Khatri A, Everill P, Bachovchin WW, Habener JF. GLP-1(32-36)amide Pentapeptide Increases Basal Energy Expenditure and Inhibits Weight Gain in Obese Mice. Diabetes. 2015;64(7):2409-19.
233. Choung JS, Lee YS, Jun HS. Exendin-4 increases oxygen consumption and thermogenic gene expression in muscle cells. Journal of molecular endocrinology. 2017;58(2):79-90.
234. Bernstein IL. Learned food aversions in the progression of cancer and its treatment. Ann N Y Acad Sci. 1985;443:365-80.
235. Jacobsen PB, Bovbjerg DH, Schwartz MD, Andrykowski MA, Futterman AD, Gilewski T, Norton L, Redd WH. Formation of food aversions in cancer patients receiving repeated infusions of chemotherapy. Behaviour research and therapy. 1993;31(8):739-48.
236. Bernstein IL, Fenner DP. Learned food aversions: heterogeneity of animal models of tumor-induced anorexia. Appetite. 1983;4(2):79-86.
237. Bernstein IL. Learned taste aversions in children receiving chemotherapy. Science. 1978;200(4347):1302-3.
238. Scalera G. Effects of conditioned food aversions on nutritional behavior in humans. Nutritional neuroscience. 2002;5(3):159-88.
239. Smith JC, Blumsack JT, Bilek FS, A.C. S, G.R. H, D.L. B. Radiation-induced taste aversion as a factor in cancer therapy. Cancer Treat Rep. 1984(68):9.
240. DeWys WD, Walters K. Abnormalities of taste sensation in cancer patients. Cancer. 1975;36(5):1888-96.
241. Hovan AJ, Williams PM, Stevenson-Moore P, Wahlin YB, Ohrn KE, Elting LS, Spijkervet FK, Brennan MT, Dysgeusia Section OCSGMAoSCiCISoOO. A systematic review of dysgeusia induced by cancer therapies. Supportive care in cancer : official journal of the Multinational Association of Supportive Care in Cancer. 2010;18(8):1081-7.
242. Hong JH, Omur-Ozbek P, Stanek BT, Dietrich AM, Duncan SE, Lee YW, Lesser G. Taste and odor abnormalities in cancer patients. The journal of supportive oncology. 2009;7(2):58-65.
243. Christensen JF, Jones LW, Andersen JL, Daugaard G, Rorth M, Hojman P. Muscle dysfunction in cancer patients. Annals of oncology : official journal of the European Society for Medical Oncology. 2014;25(5):947-58.
244. De Jonghe BC, Horn CC. Chemotherapy-induced pica and anorexia are reduced by common hepatic branch vagotomy in the rat. Am J Physiol Regul Integr Comp Physiol. 2008;294(3):R756-65.
245. Porter MH, Hrupka BJ, Langhans W, Schwartz GJ. Vagal and splanchnic afferents are not necessary for the anorexia produced by peripheral IL-1beta, LPS, and MDP. Am J Physiol. 1998;275(2 Pt 2):R384-9.
246. Bluthe RM, Michaud B, Kelley KW, Dantzer R. Vagotomy attenuates behavioural effects of interleukin-1 injected peripherally but not centrally. Neuroreport. 1996;7(9):1485-8.
247. Bluthe RM, Walter V, Parnet P, Laye S, Lestage J, Verrier D, Poole S, Stenning BE, Kelley KW, Dantzer R. Lipopolysaccharide induces sickness behaviour in rats by a vagal

mediated mechanism. Comptes rendus de l'Academie des sciences Serie III, Sciences de la vie. 1994;317(6):499-503.
248. Schwartz GJ, Plata-Salaman CR, Langhans W. Subdiaphragmatic vagal deafferentation fails to block feeding-suppressive effects of LPS and IL-1 beta in rats. The American journal of physiology. 1997;273(3 Pt 2):R1193-8.
249. Bernstein IL, Courtney L, Braget DJ. Estrogens and the Leydig LTW(m) tumor syndrome: anorexia and diet aversions attenuated by area postrema lesions. Physiol Behav. 1986;38(2):159-63.
250. Bernstein IL. Neutral mediation of food aversions and anorexia induced by tumor necrosis factor and tumors. Neuroscience and biobehavioral reviews. 1996;20(1):177-81.
251. Norgren R, Smith GP. A method for selective section of vagal afferent or efferent axons in the rat. The American journal of physiology. 1994;267(4 Pt 2):R1136-41.
252. Bernstein IL, Sigmundi RA. Tumor anorexia: a learned food aversion? Science. 1980;209(4454):416-8.
253. Van Laar ES, Desai JM, Jatoi A. Professional educational needs for chemotherapy-induced nausea and vomiting (CINV): multinational survey results from 2388 health care providers. Supportive care in cancer : official journal of the Multinational Association of Supportive Care in Cancer. 2015;23(1):151-7.
254. de Boer-Dennert M, de Wit R, Schmitz PI, Djontono J, v Beurden V, Stoter G, Verweij J. Patient perceptions of the side-effects of chemotherapy: the influence of 5HT3 antagonists. British journal of cancer. 1997;76(8):1055-61.
255. Hattori T, Yakabi K, Takeda H. Cisplatin-induced anorexia and ghrelin. Vitamins and hormones. 2013;92:301-17.
256. Hesketh PJ. Chemotherapy-induced nausea and vomiting. The New England journal of medicine. 2008;358(23):2482-94.
257. Jordan K, Jahn F, Aapro M. Recent developments in the prevention of chemotherapy-induced nausea and vomiting (CINV): a comprehensive review. Annals of oncology : official journal of the European Society for Medical Oncology. 2015;26(6):1081-90.
258. Rudd JA, Ngan MP, Wai MK. 5-HT3 receptors are not involved in conditioned taste aversions induced by 5-hydroxytryptamine, ipecacuanha or cisplatin. European journal of pharmacology. 1998;352(2-3):143-9.
259. Hickok JT, Roscoe JA, Morrow GR, King DK, Atkins JN, Fitch TR. Nausea and emesis remain significant problems of chemotherapy despite prophylaxis with 5-hydroxytryptamine-3 antiemetics: a University of Rochester James P. Wilmot Cancer Center Community Clinical Oncology Program Study of 360 cancer patients treated in the community. Cancer. 2003;97(11):2880-6.
260. Andrews PL, Horn CC. Signals for nausea and emesis: Implications for models of upper gastrointestinal diseases. Autonomic neuroscience : basic & clinical. 2006;125(1-2):100-15.
261. Parker JA, McCullough KA, Field BC, Minnion JS, Martin NM, Ghatei MA, Bloom SR. Glucagon and GLP-1 inhibit food intake and increase c-fos expression in similar appetite regulating centres in the brainstem and amygdala. Int J Obes (Lond). 2013;37(10):1391-8.
262. Kanoski SE, Fortin SM, Arnold M, Grill HJ, Hayes MR. Peripheral and Central GLP-1 Receptor Populations Mediate the Anorectic Effects of Peripherally Administered GLP-1 Receptor Agonists, Liraglutide and Exendin-4. Endocrinology. 2011;152(8):3103-12.

263. Bing C, Brown M, King P, Collins P, Tisdale MJ, Williams G. Increased gene expression of brown fat uncoupling protein (UCP)1 and skeletal muscle UCP2 and UCP3 in MAC16-induced cancer cachexia. Cancer research. 2000;60(9):2405-10.
264. Sanchis D, Busquets S, Alvarez B, Ricquier D, Lopez-Soriano FJ, Argiles JM. Skeletal muscle UCP2 and UCP3 gene expression in a rat cancer cachexia model. FEBS letters. 1998;436(3):415-8.
265. Collins P, Bing C, McCulloch P, Williams G. Muscle UCP-3 mRNA levels are elevated in weight loss associated with gastrointestinal adenocarcinoma in humans. British journal of cancer. 2002;86(3):372-5.
266. Steinert RE, Schirra J, Meyer-Gerspach AC, Kienle P, Fischer H, Schulte F, Goeke B, Beglinger C. Effect of glucagon-like peptide-1 receptor antagonism on appetite and food intake in healthy men. The American journal of clinical nutrition. 2014;100(2):514-23.
267. van Bloemendaal L, Veltman DJ, Ten Kulve JS, Groot PF, Ruhe HG, Barkhof F, Sloan JH, Diamant M, Ijzerman RG. Brain reward-system activation in response to anticipation and consumption of palatable food is altered by glucagon-like peptide-1 receptor activation in humans. Diabetes, obesity & metabolism. 2015;17(9):878-86.
268. Schirra J, Sturm K, Leicht P, Arnold R, Goke B, Katschinski M. Exendin(9-39)amide is an antagonist of glucagon-like peptide-1(7-36)amide in humans. J Clin Invest. 1998;101(7):1421-30.
269. Becskei C, Grabler V, Edwards GL, Riediger T, Lutz TA. Lesion of the lateral parabrachial nucleus attenuates the anorectic effect of peripheral amylin and CCK. Brain Res. 2007;1162:76-84.
270. Reilly S, Trifunovic R. Lateral parabrachial nucleus lesions in the rat: aversive and appetitive gustatory conditioning. Brain research bulletin. 2000;52(4):269-78.
271. Carter ME, Soden ME, Zweifel LS, Palmiter RD. Genetic identification of a neural circuit that suppresses appetite. Nature. 2013;503(7474):111-4.
272. Tkacs NC, Li J. Immune stimulation induces Fos expression in brainstem amygdala afferents. Brain research bulletin. 1999;48(2):223-31.
273. Roman CW, Derkach VA, Palmiter RD. Genetically and functionally defined NTS to PBN brain circuits mediating anorexia. Nature communications. 2016;7:11905.
274. Terawaki K, Kashiwase Y, Sawada Y, Hashimoto H, Yoshimura M, Ohbuchi K, Sudo Y, Suzuki M, Miyano K, Shiraishi S, Higami Y, Yanagihara K, Hattori T, Kase Y, Ueta Y, Uezono Y. Development of ghrelin resistance in a cancer cachexia rat model using human gastric cancer-derived 85As2 cells and the palliative effects of the Kampo medicine rikkunshito on the model. PloS one. 2017;12(3):e0173113.
275. Strasser F, Lutz TA, Maeder MT, Thuerlimann B, Bueche D, Tschop M, Kaufmann K, Holst B, Brandle M, von Moos R, Demmer R, Cerny T. Safety, tolerability and pharmacokinetics of intravenous ghrelin for cancer-related anorexia/cachexia: a randomised, placebo-controlled, double-blind, double-crossover study. British journal of cancer. 2008;98(2):300-8.
276. Garcia JM, Garcia-Touza M, Hijazi RA, Taffet G, Epner D, Mann D, Smith RG, Cunningham GR, Marcelli M. Active ghrelin levels and active to total ghrelin ratio in cancer-induced cachexia. J Clin Endocrinol Metab. 2005;90(5):2920-6.
277. Baatar D, Patel K, Taub DD. The effects of ghrelin on inflammation and the immune system. Mol Cell Endocrinol. 2011;340(1):44-58.
278. Villars FO, Pietra C, Giuliano C, Lutz TA, Riediger T. Oral Treatment with the Ghrelin Receptor Agonist HM01 Attenuates Cachexia in Mice Bearing Colon-26 (C26) Tumors. International journal of molecular sciences. 2017;18(5).

279. Ribaudo JM, Cella D, Hahn EA, Lloyd SR, Tchekmedyian NS, Von Roenn J, Leslie WT. Re-validation and shortening of the Functional Assessment of Anorexia/Cachexia Therapy (FAACT) questionnaire. Quality of life research : an international journal of quality of life aspects of treatment, care and rehabilitation. 2000;9(10):1137-46.
280. DeBoer MD, Zhu XX, Levasseur P, Meguid MM, Suzuki S, Inui A, Taylor JE, Halem HA, Dong JZ, Datta R, Culler MD, Marks DL. Ghrelin treatment causes increased food intake and retention of lean body mass in a rat model of cancer cachexia. Endocrinology. 2007;148(6):3004-12.
281. Hanada T, Toshinai K, Date Y, Kajimura N, Tsukada T, Hayashi Y, Kangawa K, Nakazato M. Upregulation of ghrelin expression in cachectic nude mice bearing human melanoma cells. Metabolism: clinical and experimental. 2004;53(1):84-8.
282. Northrup R, Kuroda K, Duus EM, Barnes SR, Cheatham L, Wiley T, Pietra C. Effect of ghrelin and anamorelin (ONO-7643), a selective ghrelin receptor agonist, on tumor growth in a lung cancer mouse xenograft model. Support Care Cancer. 2013;21(9):2409-15.
283. Wang W, Andersson M, Iresjo BM, Lonnroth C, Lundholm K. Effects of ghrelin on anorexia in tumor-bearing mice with eicosanoid-related cachexia. International journal of oncology. 2006;28(6):1393-400.
284. Lundholm K, Gunnebo L, Korner U, Iresjo BM, Engstrom C, Hyltander A, Smedh U, Bosaeus I. Effects by daily long term provision of ghrelin to unselected weight-losing cancer patients: a randomized double-blind study. Cancer. 2010;116(8):2044-52.
285. Garcia JM, Boccia RV, Graham CD, Yan Y, Duus EM, Allen S, Friend J. Anamorelin for patients with cancer cachexia: an integrated analysis of two phase 2, randomised, placebo-controlled, double-blind trials. Lancet Oncol. 2015;16(1):108-16.
286. Takayama K, Katakami N, Yokoyama T, Atagi S, Yoshimori K, Kagamu H, Saito H, Takiguchi Y, Aoe K, Koyama A, Komura N, Eguchi K. Anamorelin (ONO-7643) in Japanese patients with non-small cell lung cancer and cachexia: results of a randomized phase 2 trial. Supportive care in cancer : official journal of the Multinational Association of Supportive Care in Cancer. 2016;24(8):3495-505.
287. Neary NM, Small CJ, Wren AM, Lee JL, Druce MR, Palmieri C, Frost GS, Ghatei MA, Coombes RC, Bloom SR. Ghrelin increases energy intake in cancer patients with impaired appetite: acute, randomized, placebo-controlled trial. J Clin Endocrinol Metab. 2004;89(6):2832-6.
288. Garcia JM, Polvino WJ. Pharmacodynamic hormonal effects of anamorelin, a novel oral ghrelin mimetic and growth hormone secretagogue in healthy volunteers. Growth hormone & IGF research : official journal of the Growth Hormone Research Society and the International IGF Research Society. 2009;19(3):267-73.
289. Lasseter KC, Shaughnessy L, Cummings D, Pezzullo JC, Wargin W, Gagnon R, Oliva J, Kosutic G. Ghrelin agonist (TZP-101): safety, pharmacokinetics and pharmacodynamic evaluation in healthy volunteers: a phase I, first-in-human study. Journal of clinical pharmacology. 2008;48(2):193-202.
290. Nagaya N, Kojima M, Uematsu M, Yamagishi M, Hosoda H, Oya H, Hayashi Y, Kangawa K. Hemodynamic and hormonal effects of human ghrelin in healthy volunteers. Am J Physiol Regul Integr Comp Physiol. 2001;280(5):R1483-7.
291. Wren AM, Seal LJ, Cohen MA, Brynes AE, Frost GS, Murphy KG, Dhillo WS, Ghatei MA, Bloom SR. Ghrelin enhances appetite and increases food intake in humans. J Clin Endocrinol Metab. 2001;86(12):5992.

292. Bai Y, Hu Y, Zhao Y, Yu X, Xu J, Hua Z, Zhao Z. Anamorelin for cancer anorexia-cachexia syndrome: a systematic review and meta-analysis. Supportive care in cancer : official journal of the Multinational Association of Supportive Care in Cancer. 2017.
293. Morley JE, Thomas DR, Wilson MM. Cachexia: pathophysiology and clinical relevance. Am J Clin Nutr. 2006;83(4):735-43.

11 Curriculum vitae

Tito Borner

1. PERSONAL DETAILS AND CONTACT

Date of birth	22.02.1986	Kalkbreite 59
Place of birth	Locarno	8057 Zurich
Citizenship	Swiss	Switzerland
Status	Lone	Phone: +041 78 871 66 57
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2. EDUCATION

Since 05/2011	**PhD Student** at the Institute of Veterinary Physiology, University of Zurich (UZH), Switzerland Running title: "Neuronal mechanisms involved in cancer anorexia cachexia syndrome and the evaluation of possible therapeutic approaches" PhD Program: Integrative Molecular Medicine (imMed) Supervision: Prof. Dr. PhD Thomas Riediger
04/2010 – 04/2011	**Master of Science in Biology** at the University of Zurich (UZH), Switzerland Major field: Human Biology **Master's Thesis** at the Veterinary Physiology Institute of Zurich. Title: "The anorectic endotoxin LPS inhibits ghrelin-excited neurons of the arcuate nucleus via iNOS-dependent nitric oxide signaling" Supervision: Prof. Dr. med. vet. Thomas A. Lutz and Prof. Dr. PhD Thomas Riediger
10/2005 – 03/2010	**Bachelor of Science in Biology** at the University of Zurich (UZH), Switzerland
09/2001 – 07/2005	**Swiss High School** (Liceo Cantonale di Locarno, LiLo), Locarno, Ticino, Switzerland. Major subject: biology, chemistry and physics
09/1997 – 06/2001	**Swiss Middle School** (Scuole Medie Losone), Losone, Ticino, Switzerland

3. EMPLOYMENT HISTORY AND WORK EXPERIENCE

Since 05/2011	PhD Student at the Institute of Veterinary Physiology, University of Zurich, Prof. Dr. med. vet. Thomas A. Lutz laboratory. Supervision: Prof. Dr. PhD Thomas Riediger.

04/2010 – 04/2011	Master's project at the Institute of Veterinary Physiology University of Zurich, Prof. Dr. med. vet. Thomas A. Lutz laboratory. Supervision: Prof. Dr. med. vet. Thomas A. Lutz and Prof. Dr. PhD Thomas Riediger.
2004 – 2010	Voluntary paramedic at the ambulance sanitary service in Locarno.
2002 – 2014	Swimming instructor in a primary school program during the summer season.

4. LANGUAGE SKILLS

Italian	Native language
German	Good knowledge
English	Good knowledge
French	Intermediate knowledge
Spanish	Basic knowledge

5. APPROVED RESEARCH PROJECT

FK-14-047: Candoc Forschungskredit, University of Zurich, July 2014 - June 2015
Title: The relevance of central GLP-1 signaling in the mediation of cancer anorexia (58'000 CHF).

6. PRIZES AND AWARDS

Best poster award at 9th Symposium of the Zurich Centre for Integrative Human Physiology, Zurich, Switzerland, August 2013.

Best poster award at 10th Symposium of the Zurich Centre for Integrative Human Physiology, Zurich, Switzerland, August 2014.

7. PUBLICATIONS

Borner, T, Pinkernell, S, Lutz TA, Riediger, T. Lipopolysaccharide inhibits ghrelin-excited neurons of the arcuate nucleus and reduces food intake via central nitric oxide signaling. Brain, Behavior, and Immunity. 2012.

Borner T, Loi L, Pietra C, Giuliano C, Lutz TA, Riediger T. The ghrelin receptor agonist HM01 mimics the neuronal effects of ghrelin in the arcuate nucleus and attenuates anorexia-cachexia syndrome in tumor-bearing rats. American Journal of Physiology Regulatory, Integrative and Comparative Physiology. 2016.

Liberini CG, **Borner T**, Boyle CN, Lutz TA. The satiating hormone amylin enhances neurogenesis in the *area postrema* of adult rats. Molecular Metabolism. 2016.

Borner T, Arnold M, Ruud J, Breit SN, Langhans W, Lutz TA, Blomqvist A, Riediger T. Anorexia-cachexia syndrome in hepatoma tumor-bearing rats requires the area postrema but not vagal afferents and is paralleled by increased MIC-1/GDF15. Journal of Cachexia, Sarcopenia and Muscle. 2016.

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